

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29239 A2

(51) International Patent Classification⁷: C12N 15/82,
15/29, C07K 14/415, C12N 5/10, C12Q 1/68, A01H 5/00

(74) Agents: KREMER, Simon, M. et al.; Mewburn Ellis,
York House, 23 Kingsway, London WC2B 6HP (GB).

(21) International Application Number: PCT/GB00/03930

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 12 October 2000 (12.10.2000)

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

Published:

— Without international search report and to be republished
upon receipt of that report.

(26) Publication Language: English

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(30) Priority Data:
9924483.2 15 October 1999 (15.10.1999) GB

(71) Applicant (for all designated States except US): PLANT
BIOSCIENCE LIMITED [GB/GB]; Norwich Research
Park, Colney Lane, Norwich, Norfolk NR4 7UH (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BENDAHMANE,
Abdelhafid [MA/FR]; INRA-URGV, 2, rue Gaston
Crémieux, Case postale 5708, F-91057 Evry Cedex (FR).
BAULCOMBE, David, Charles [GB/GB]; 63 Caernar-
von Road, Norwich, Norfolk NR2 3HY (GB).



A2

(54) Title: MODIFIED RESISTANCE GENES

WO 01/29239

(57) Abstract: Disclosed are processes for modifying the activation characteristics of a first polypeptide having an amino acid sequence which includes a nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain, which first polypeptide mediates a cellular response leading to pathogen resistance and/or cell death or dysfunction in response to an elicitor (e.g. apoptosis regulator or plant resistance polypeptide such as Rx), the process comprising the step of introducing a modification to the amino acid sequence of the first polypeptide such as to produce an auto-activator polypeptide which is capable of activation in the absence of the elicitor. Preferred modifications include those which decreases the net negative charge of the NB-ARC region, particularly in or around GPLP, CFLY or MHD motifs. Other preferred modifications are such that the auto-activator polypeptide is artificially dimerized, or is dimerized under predefined conditions in response to a dimerizing effector agent which is not the elicitor. Also disclosed are processes for producing such auto-activator polypeptides and nucleic acids encoding the same, plus various methods and materials for use in such processes, and methods of using the products e.g. for influencing or affecting a cellular response in a plant.

MODIFIED RESISTANCE GENESTECHNICAL FIELD

5 The present invention relates to modified resistance or other genes, for instance for use in plants, and methods and materials for producing the genes.

PRIOR ART

10 Many disease resistance (R) genes in plants encode proteins with characteristic sequence motifs including a nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain (Hammond-Kosack and Jones, 1997). Genetic evidence indicates that these NBS-LRR
15 proteins interact, directly or indirectly, with pathogen components. This recognition process then initiates signalling pathways leading to cell death and disease resistance (Staskawicz et al., 1995). In general terms the R protein is considered as a receptor that interacts with a ligand, an elicitor, from the
20 pathogen. In common with other receptors it is generally considered that R proteins have a modular structure with separate recognition and signalling domains.

It is believed that the recognition domain may involve the C
25 terminal LRR domain. Sequence analysis of related R genes indicates that this is the most variable region of the protein and that it is under selection to diverge (Meyers et al., 1998).

The N terminal domains of the R proteins have been implicated in
30 signalling through the identification of sequence motifs. These motifs, referred to collectively as the NB-ARC domain, include the Ap-ATPase region in which there are five signature motifs that differentiate these proteins from other nucleotide binding proteins (Aravind et al., 1999).

35

Two other motifs, referred to as 'CFLY' and 'MHD,' are also included in the NB-ARC domain (Hammond-Kosack and Jones, 1997; van

der Biezen and Jones, 1998).

Functional analysis of recombinant R proteins is also consistent with these views, although there is an example in which these 5 artificial R proteins do not have the recognition specificity of either progenitor: thus it is possible that the N terminal parts of the protein also participate in or influence the recognition process (Ellis et al., 1999).

10 DISCLOSURE OF THE INVENTION

The present inventors have investigated the activation of NBS-LRR R proteins in plants. The inventors have succeeded in modifying the activation characteristics of these R proteins such that they 15 were able to activate a resistance response in the absence of their natural elicitors. These gain of function modifications included, *inter alia*, point mutations, for instance in the highly conserved NB-ARC domain, and to a lesser extent in the LRR domain, and also artificial dimerisation of the R proteins. Modified NBS- 20 LRR R proteins having these characteristics have not previously been disclosed in the art. This decoupling of the R response from its natural elicitor has potential utility, *inter alia*, in developing novel pathogen responsive plants.

25 Thus in general terms, the invention provides processes for modifying the activation characteristics of a (first) polypeptide capable of conferring elicitor-dependent activation of resistance response against a pathogen (i.e. an R protein). In essence the process comprises the step of modifying the sequence of the R 30 protein which displays such elicitor-dependent activation, such that activation of the resistance response can be achieved in the absence of the elicitor. 'Elicitor dependent' in this context means that under normal conditions, for instance at cellular levels found *in planta* under its natural promoter, the R gene does 35 not activate a resistance response in the absence of the pathogen or an elicitor therefrom. In contrast the modified activation characteristic will be automatic (e.g. a so called 'auto-

activator' R protein which is permanently switched on by mutation or dimerisation) or will be based upon artificial dimerisation under predefined conditions (e.g. an R protein can be dimerised and hence activated in response to a non-native dimerising agent).

5

Interestingly the results have implications not only for the use of modified plant R proteins, but also for certain mammalian proteins involved in apoptosis. For instance the R proteins share the NB-ARC domain with the CED4 (*Caenorhabditis elegans*) and APAF-1 (human) regulators of apoptosis (van der Biezen and Jones, 1998; Aravind et al., 1999). Disease resistance in plants is often characterised by cell death at, and around, the site of pathogen inoculation and the similarity of R proteins and apoptosis regulators means that the results herein have

10 implications for the latter. Thus the skilled person will appreciate that the aspects of the invention disclosed herein apply correspondingly to mammalian apoptosis regulators in mammalian cells.

15 Thus in one aspect the present invention relates to a process for producing (or identifying, or isolating) a modified NB-ARC protein, which comprises the steps of:

(i) selecting a (first) NB-ARC domain protein which is not autonomously activated,

20 (ii) modifying the NB-ARC domain such as to produce a protein which is capable of autonomously activating a cellular response leading to cell death or dysfunction (e.g. an apoptosis response, or HR). The modified protein is optionally screened to confirm this activity.

25

The inventors used as their exemplary system mutation analysis of Rx from potato. Rx encodes an NBS-LRR protein that mediates recognition of the coat protein of potato virus X (PVX) leading to virus resistance. The Rx-mediated resistance against PVX is

30 thought to conform to an elicitor-receptor model. According to the model there are two phases in the Rx resistance mechanism: a recognition phase that is believed to be highly specific for the

potato virus X coat protein (CP) elicitor and a response phase that prevents accumulation of a broad spectrum of plant viruses, including those taxonomically unrelated to PVX. Further details of Rx and its properties may be found in PCT/GB99/01182 (Plant Bioscience Limited), which details, inasmuch as they may be required to support the present invention (e.g. by reference to sequences which of Rx or which are homologous to Rx) are incorporated herein by reference.

10 There is a very high degree of similarity between Rx and a subclass of NBS-LRR resistance proteins represented by Rps2, Rpm1 and Prf (Jones and Jones, 1997). These *Arabidopsis* and tomato proteins contain a putative four to six heptad amphipathic leucine zipper (LZ) motif at the N-terminus (Jones and Jones, 1997). As 15 in the other *R* gene products, the putative NBS domain of Rx comprises three motifs: kinase 1A or 'P-loop', kinase 2, and kinase 3a. In Rx, the putative NBS is followed by a domain that includes GLPL, CFLY and the MHD motifs. These motifs are characteristic of all NBS-LRR *R* gene products thus far identified 20 (Hammond-Kosack and Jones, 1997; van der Biezen and Jones, 1998). A putative LRR domain of Rx comprises 14-16 imperfect copies of the LRR motif. This motif shows a good match to the cytoplasmic LRR consensus sequence motif (Jones and Jones, 1997) and most closely resembles the LRR domain of the tomato Prf protein 25 (Salmeron et al., 1996). Generally speaking, however, the sequence conservation between the Rx and other disease resistance genes is mostly in the NBS domain in the N terminal part of Rx.

It is highly likely that the findings disclosed herein will apply 30 generally to other NB-ARC domain *R* proteins which will together provide means for conferring resistance against bacteria, fungi and invertebrates (e.g. insects such as aphids). For instance Rx resistance response is effective against viruses that are unrelated to PVX (Bendahmane et al., 1995) and the Rx homologue in 35 BAC111 (see PCT/GB99/01182, Plant Bioscience Limited) is a nematode resistance gene (Bendahmane and Baulcombe, 1999; Rouppe van der Voort et al., 1999). Other NB-ARC domain containing *R*

genes include the root knot nematode resistance gene 'MI' from tomato, which also confers resistance against potato aphid (see Milligan et al, 1998 Plant Cell 10, 1307-1319; Rossi et al, 1998 Proc Natl Acad Sci USA 95, 9750-9754. Also the 'N' gene which 5 gives resistance against TMV (see Whitham et al, 1994 Cell 78, 1105-1115).

The modified resistance proteins disclosed herein will have utility, *inter alia*, in conferring resistance in response to non-10 natural agents or stimuli, and also for investigating resistance response pathways and protein interactions e.g. with activators and repressors.

More specifically there are several ways that disease resistance 15 in plants could be achieved by expression of these modified R proteins:

(i) Low level constitutive expression of auto-activators. High 20 level expression would lead to HR (i.e. death) (Gilbert et al., 1998). However low level expression could lead to activation of the primary resistance response that is HR independent.

(ii) Expression of auto-activators under control of a pathogen induced promoter.

25 (iii) Expression of the auto-activators from a viral amplicon (Angell and Baulcombe, 1997). It might be expected that the amplicon would mediate expression of the auto-activator until resistance was activated at a level that prevented further virus 30 accumulation. Because auto-activator expression in this system would be mediated by virus accumulation the system would be self regulating.

(iv) Use of auto-activators in a GEAR strategy (see WO 95/31564, 35 Gatsby Charitable Foundation). The auto-activator would be inactivated by insertion of a transposon. Movement of the transposon out of the auto-activator would lead to resistance/HR

in cells or small sectors of the plant. Salicylic acid or other extracellular signals might then mediate systemic resistance.

5 (v) Expression from an inducible promoter so that activation of the HR/resistance response would be activated following treatment with a promoter inducing agent. For example, if Rx was expressed under control of the dex or alcohol inducible promoters the Rx response would be inducible by either dex or alcohol (Aoyama and Chua, 1997).

10

(vi) Auto-activators could also be used to control development. For example, if the auto-activators were expressed under control of a pollen specific promoter there would be death of the pollen cells and male sterility. This could also be used as a strategy 15 in developing (e.g. trees) that did not flower.

20 (vii) R proteins could be expressed modified such that they could be dimerised in the presence of a specific dimerizing agent, which in turn could be expressed under the control of an inducible promoter activated by a particular pathogen. Likewise dimerised (e.g. via a linker) or tandem repeat R proteins, could themselves be expressed under an inducible promoter.

25 Some of these aspects of the invention will now be discussed in more detail.

Thus in a one aspect of the present invention there is provided a process for modifying the activation characteristics of a first polypeptide having an amino acid sequence which includes a 30 nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain, which first polypeptide mediates a cellular response leading to pathogen resistance and\or cell death or dysfunction in response to an elicitor, the process comprising the step of introducing a modification to the amino acid sequence of the first 35 polypeptide such as to produce an auto-activator polypeptide which is capable of activation in the absence of the elicitor.

Thus this aspect provides a process for producing (or identifying, or isolating) a modified R protein which is capable of activating a resistance response in the absence of a pathogen (or elicitor therefrom) the process comprising the steps of:

- 5 (i) selecting an NB-ARC domain R protein which displays elicitor-dependent activation,
- (ii) modifying the amino acid sequence of the protein such as to nullify the elicitor dependence.
- 10 Optionally this is followed by the further step of screening the modified R protein for its autoactivation properties.

As described above, the 'elicitor dependent' protein prior to modification refers to the protein's characteristic under normal conditions. For instance Rx, when expressed under its own promoter *in vivo* is an elicitor-dependent R protein. By modifying the elicitor dependence, the Rx protein can be 'switched on' even in the elicitor's absence (although this does not imply that the protein may not be switched in its presence). Thus in the Examples below, modified Rx proteins have been produced which, in the absence of the PVX coat protein, or other homologous 'natural' elicitors, lead to activation of an Rx resistance response. Generally the modification will be achieved by expression from a modified nucleic acid sequence, as described in more detail hereinafter.

Again generally speaking, in order to screen for auto-activator function, a comparison is made between:

- (i) the unmodified R protein (in absence of 'natural' elicitor) which will give a negative resistance response, and
- 30 (ii) the modified auto-activator R protein (in absence of 'natural' elicitor) which will give a positive resistance response.
- 35 Optionally other controls are used, namely:
 - (iii) the unmodified R protein in presence of 'natural' elicitor, which will give a positive response, and,

(iv) non-autoactivating mutations which in presence of 'natural' elicitor, which may give a negative response.

The analysis may be done using transient or stable expression of
5 the appropriate proteins e.g. R protein and elicitor in plants. The resistance response, as shown in the Examples, may be observed directly (e.g. challenge of appropriate pathogen, or related reporter construct) or may be inferred from an associated resistance effect e.g. a hypersensitive response (HR) resulting in
10 necrosis or other cell damage (see WO 95/31564, Gatsby Charitable Foundation, for a general discussion of HR). Example methods for testing R gene activity can be found in the following publications: bacterial (Grant et al, 1995); fungal (Dixon et al, 1996; Jones, 1994; Thomas et al, 1997); nematode and viral
15 (Whitham et al, 1994). These can be modified as required in the light of the present disclosure in order to detect the autoactivating mutations. Typically, activity is tested ultimately by complementation of trait in a plant. This can be achieved by coupling the putative autoactive variant to a promoter and
20 terminator for expression in plants and transforming it into a 'susceptible' plant that lacks a given resistance trait. The activity of the auto-activator is then confirmed by challenge with the appropriate pathogen.

25 The formats described above, to assess R protein autoactivation in the absence of known elicitors, themselves form a further aspect of the present invention. In particular the processes for establishing a decoupling of a gene for gene compatibility between elicitor and R gene, are characterised in that they include the
30 steps of:

- (a) expressing the unmodified R protein in a system in the absence of 'natural' elicitor, and,
- (b) expressing the modified auto-activator R protein in the system in the absence of 'natural' elicitor,
- 35 (c) observing the system in each case for a resistance response,
- (d) correlating the result of the observation made in (c) with the autoactivating effect of the modification.

Results obtained with Rx suggest that the N terminal region (i.e. without the LRR) is the signalling domain and that activation of the Rx resistance response is mediated by dimerization.

5 Thus although the results herein show that point mutations may be made in the LRR, mutations in the NB-ARC region, particularly close to or in the MHD or CFLY regions are a particularly effective way of inducing an autoactivated R protein response. Thus residues within these regions (or within distances less than 10 20, 15, 10, 9, 8, 7, more preferably 6, 5, 4, 3, 2 or 1 residue(s) of them) are preferred targets for modification. The identification of corresponding regions in other proteins may be readily achieved by those skilled in the art on the basis of their own general knowledge and the disclosure herein (e.g. Figure 3A).

15

Optionally the LRR region may be deleted, and the mutations made elsewhere.

Preferably the effect is achieved by modifying the identity of 20 only 1,2,3,4,5, 10 or more amino acids. However, naturally, the invention also embraces multiple mutations including multiple mutations each having an auto-activator effect, possibly in conjunction with mutations made for quite different reasons. Put another way, there is no requirement that the initial selected 25 sequence is 'wild-type' or naturally occurring, or even full-length (although this may be preferred) provided that mutations are introduced which have the effects discussed above.

Interestingly most of the mutations described herein result in a 30 net increase in the charge of the Rx protein (see Figure 3B). Thus mutations which have this effect are also preferred e.g. substitution of 'acidic' amino acids (such as Glu and Asp) for neutral, or basic ones (such as Arg, Lys, His), or neutral ones for basic ones, in accordance with the pKa values of their side 35 chains. Put another way, the desired mutation may be one which decreases the net negative charge of the NB-ARC region (or regions therein as discussed above) thereby modulating or otherwise

inhibiting an electrostatic interaction with a more positive binding partner e.g. a repressor.

Most preferred mutations are correspond to, or are identical with, 5 any one or more of those shown in Figure 3B. By 'correspond to' is meant an alteration of an equivalent amino acid i.e. one which aligns with any of the depicted amino acids on a sequence line-up, such are shown in the Tables herein.

10 In another embodiment the modification comprises the incorporation of a heterologous dimerization-enabling sequence into the selected protein. Such an enabling sequence will permit dimerization of the protein in which it is incorporated in the presence of a dimerization effector agent. Examples are given in Experimental 15 Procedures section below. Generally the enabling sequence will be added to the R protein (or portion thereof) as a fusion.

All such sequences incorporating such mutations are, for brevity, referred to hereinafter as 'auto-activators'.

20 In one aspect of the present invention there is disclosed an auto-activator R polypeptide obtainable by the processes described above.

25 According to a further aspect of the present invention there is provided a nucleic acid molecule encoding an auto-activator R protein (or polypeptide) which has been modified in the terms discussed above e.g. is capable of initiating a resistance response against a pathogen even in the absence of its natural 30 elicitor. The expression product of these nucleic acids, and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions, are also encompassed.

35 Nucleic acid molecules according to the present invention may be provided in recombinant form or free or substantially free of nucleic acid or genes of the species of interest or origin other

than the sequence encoding a polypeptide with the required function. The nucleic acid molecules (and their encoded polypeptide products) may also be (i) isolated and/or purified from their natural environment (although not necessarily in pure 5 form *per se*), or (ii) in substantially pure or homogeneous form.

Nucleic acid according to the present invention may include cDNA or RNA but will be wholly or at least partially synthetic ('constructs'). Where a DNA sequence is specified, e.g. with 10 reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Also encompassed is the complement of the various disclosed 15 sequences, which may be used in probing experiments, or in down-regulation of the sequence. The 'complement' in each case is the same length as the reference, but is 100% complementary thereto whereby by each nucleotide is base paired to its counterpart i.e. G to C, and A to T or U.

20 Particular polypeptides include the 'auto-activator' sequences labelled 193, 25, 32, 39, 7, 72 in Table III below. Nucleic acids include those encoding all or a functional (autoactivated) part of these sequences. Nucleic acids of the invention include 25 those shown in Table II.

The autoactivating R gene activity can be tested by methods described herein, or analogous to those, as appropriate to the nature of the resistance being investigated.

30 Where the terms similarity, homology or identity are used herein they can be established e.g. using the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the 35 standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Comparisons

herein have used DNASTAR software using the CLUSTAL method with PAM250 residue weight table (gap penalty 10, gap length 10).

Homology (or similarity, or identity) may be at the nucleotide sequence and/or the expressed amino acid sequence level.

Preferably, the nucleic acid and/or amino acid sequence shares homology with the NB-ARC coding sequences herein preferably at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology.

Homology may be over the full-length of the relevant sequence shown herein, or may more preferably be over a contiguous sequence of about or greater than about e.g. 20, 100, 200, 300, 500, 600 or more amino acids or codons, compared with the relevant amino acid sequence or nucleotide sequence as the case may be.

15

Similarity to the disclosed sequences may be established using probes based on the sequences e.g. in southern blotting.

Preliminary experiments may be performed by hybridising under low stringency conditions. For example, hybridizations may be performed, according to the method of Sambrook et al. (below) using a hybridization solution comprising: 5X SSC (wherein 'SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): $T_m = 81.5^\circ C + 16.6 \log [Na^+] + 0.41 (\% G+C) - 0.63 (\% formamide) - 600/\#bp$ in duplex. As an illustration of the above formula, using $[Na^+] = [0.368]$ and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of

a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous 5 to the nucleic acid sequence of the present invention. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na₂HPO₄, 10 pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 15 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS. Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include amplification 20 using PCR (including, where appropriate, RACE PCR), RN'ase protection and allele specific oligonucleotide probing.

In one embodiment, the invention provides autoactivating homologous variants of the Rx sequences provided, which may for 25 instance comprise additional mutations, or be based on autoactivating derivatives of naturally occurring Rx homologues such as other R proteins including the NB-ARC region, allelic variants, paralogues, or orthologues.

30 There are believed to be more than 20 homologues of Rx in the potato genome alone. It is likely that one or more of these homologues are R genes against viruses, fungi, bacteria or nematodes. These can be isolated or identified by methods disclosed in PCT/GB99/01182 (Plant Bioscience Limited). They may 35 be modified in the terms described above to provide auto-activator resistance. For instance work done by the present inventors indicates that Rx2 can be correspondingly autoactivated by

introducing a D to V substitution in the MHD region (see Example 1). These autoactivated an HR with similar kinetics to the corresponding Rx mutant (data not shown).

- 5 Preferably the nucleic acid molecule which is the autoactivating mutant is generated either directly or indirectly (e.g. via one or amplification or replication steps) from an original nucleic acid corresponding to the NB-ARC protein.
- 10 Changes to a sequence, to produce a mutant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide.

15

In addition to one or more changes within the Rx sequence to produce autoactivation, a variant nucleic acid may encode an amino acid sequence including additional amino acids at the C-terminus and/or N-terminus, for instance to facilitate dimerization, or to 20 actually generate a dimer (which is autoactivated).

Specifically included are parts or fragments (however produced) corresponding to portions of the sequences provided, and which encode derivative polypeptides having autoactivating biological 25 activity.

Other changes to the sequence (apart from autoactivating mutations) may be desirable for a number of reasons, including introducing or removing the following features: restriction 30 endonuclease sequences; codon usage; other sites which are required for post translation modification; cleavage sites in the encoded polypeptide; motifs in the encoded polypeptide for glycosylation, lipoylation etc. Leader or other targeting sequences may be added to the expressed protein to determine its 35 location following expression. All of these may assist in efficiently cloning and expressing an autoactive polypeptide in recombinant form (as described below).

Changes, whether for the purpose of autoactivation or otherwise, may include conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or 5 methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine.

Oligonucleotides for use in PCR mutagenesis include those shown in 10 the Examples below, and may be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length may be preferred. Those skilled in the art are well versed in the design 15 of primers for use processes such as PCR.

In one aspect of the present invention, the nucleic acid described above is in the form of a recombinant and preferably replicable vector.

20 "Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic 25 host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Specifically included are shuttle vectors by which is meant a DNA 30 vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eucaryotic (e.g. higher plant, mammalian, yeast or fungal cells).

35 A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic

acid into cells for recombination into the genome.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other 5 regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be 10 under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream 15 (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to 20 be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operatively 25 linked to a nucleotide sequence provided by the present invention, such as an auto-activator Rx mutant.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene 30 expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular 35 Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (see above), sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Particularly of interest in the present context are plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Bevan (Nucl. Acids Res. 12, 8711-8721 (1984)) and Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

In one embodiment of this aspect of the present invention provides a gene construct, preferably a replicable vector, comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired

phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

5 As described above, preferred inducible promoters may be those which are activated by either (i) a pathogen (particularly one which does not provide the 'natural' elicitor of the R protein but which is nonetheless affected by the resistance response) or (ii) an artificial inducer such as ethanol which can be readily applied

10 by human intervention. For instance the GST-II-27 gene promoter, which has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of

15 genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues. Other promoters include the patatin promoter (tubers), ubiquitin promoter (wheat embryos).

25 The promoter may include one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression.

Another possibility is that an artificially dimerizable R protein

30 is operably linked to a constitutive promoter, and the same or a different construct is provided in which the dimerizing effector is operably linked to an appropriate inducible promoter.

Thus the vectors of the present invention may include the

35 autoactivating gene, in addition to various sequences required to give them replicative, integrative and/or expression functionality, including ancillary dimerization effectors. Such

vectors can be used, for instance, to make plants into which they are introduced resistant to plant pathogens.

In addition to the vectors and constructs above, the present
5 invention also provides methods comprising introduction of these constructs discussed above (such as vectors) into a host cell and/or induction of expression of a construct within a plant cell, by application of a suitable stimulus, an effective exogenous inducer.

10

The vectors described above may be introduced into hosts by any appropriate method e.g. conjugation, mobilisation, transformation, transfection, transduction or electroporation, as described in further detail below.

15

In a further aspect of the invention, there is disclosed a host cell containing nucleic acid or a vector according to the present invention, especially a plant or a microbial cell.

20 Thus DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616)
25 microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant 30 Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

35 *Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of

stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* 6, 1072-1074; Zhang, et al. (1988) *Plant Cell Rep.* 7, 379-384; Zhang, et al. (1988) *Theor Appl Genet* 76, 835-840; Shimamoto, et al. 5 (1989) *Nature* 338, 274-276; Datta, et al. (1990) *Bio/Technology* 8, 736-740; Christou, et al. (1991) *Bio/Technology* 9, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao, et al. (1992) *Plant Cell Rep.* 11, 585-591; Li, et al. (1993) *Plant Cell Rep.* 12, 250-255; Rathore, et 10 al. (1993) *Plant Molecular Biology* 21, 871-884; Fromm, et al. (1990) *Bio/Technology* 8, 833-839; Gordon-Kamm, et al. (1990) *Plant Cell* 2, 603-618; D'Halluin, et al. (1992) *Plant Cell* 4, 1495-1505; Walters, et al. (1992) *Plant Molecular Biology* 18, 189-200; Koziel, et al. (1993) *Biotechnology* 11, 194-200; Vasil, I. K. 15 (1994) *Plant Molecular Biology* 25, 925-937; Weeks, et al. (1993) *Plant Physiology* 102, 1077-1084; Somers, et al. (1992) *Bio/Technology* 10, 1589-1594; WO92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient alternative transformation method in monocots 20 (Hiei et al. (1994) *The Plant Journal* 6, 271-282).

Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be 25 employed to enhance the efficiency of the transformation process, eg bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233). 30 The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a 35 transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the

choice of technique for plant regeneration.

If desired, selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as 5 resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

Thus a further aspect of the present invention provides a method 10 of transforming a plant cell involving introduction of a vector comprising a nucleic acid of the present invention into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

15 The invention further encompasses a host cell transformed with nucleic acid or a vector according to the present invention, especially a plant or a microbial cell. In the transgenic plant cell (i.e. transgenic for the nucleic acid in question) the 20 transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. There may be more than one heterologous nucleotide sequence per haploid genome.

The term "heterologous" is used broadly in this aspect to indicate 25 that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A heterologous gene may be additional to a corresponding endogenous 30 gene (which, clearly, will not have been modified to be an auto-activator). Nucleic acid heterologous, or exogenous or foreign, to a plant cell will be non-naturally occurring in cells of that type, variety or species. Thus the heterologous nucleic acid may comprise a coding sequence of or derived from a particular type of 35 plant cell or species or variety of plant, modified and placed within the context of a plant cell of a different type or species or variety of plant.

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are

5 reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

10 The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, et al. (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

15 Plants which include a plant cell according to the invention are also provided, along with clones, selfed or hybrid progeny and other descendants. A plant according to the present invention may 20 be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights.

25 In addition to the plants, the present invention embraces any part of the plants such as cuttings etc. The invention also provides a plant propagule from such a plant, that is any part which may be used in reproduction or propagation, sexual or asexual, including seed and so on.

30 Antibodies may be raised to a purified polypeptides or peptide by any method known in the art (for an overview see e.g. "Immunology - 5th Edition" by Roitt, Brostoff, Male: Pub 1998 - Mosby Press, London).

35 The invention further provides a method of influencing or affecting a resistance trait in a plant, whereby the method includes the step of causing or allowing expression of a

heterologous nucleic acid sequence as discussed above within cells of the plant. Preferably the invention provides a method which includes expressing the nucleic acid of the invention within the cells of a plant (thereby producing the encoded polypeptide), 5 following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Generally such a method may be used to introduce pathogen resistance into the plant whereby resistance (e.g. ER or HR) is triggered by contact with an appropriate non-natural (i.e. not the original, natural, elicitor) 10 inducer. Broadly speaking the inducer may be encoded directly by the invading pathogen. Alternatively it may be expressed by a separate construct or transgene which is itself triggered or upregulated by the pathogen infection.

15 It is also possible to use the disclosure herein in respect of the activation of cell death in animal cells.

Thus processes for producing (or identifying, or isolating) a modified apoptosis regulator protein which is capable of 20 activating an apoptosis response in a mammalian cell may comprise the steps of:

- (i) selecting an NB-ARC domain apoptosis protein which displays elicitor-dependent activation,
- (ii) modifying the amino acid sequence of the NB-ARC domain of the 25 protein such as to generate an auto-activator protein.

The modification may be in similar regions as those discussed above. For example, by introducing the MHD to MHV mutation (as in AT39 and AT193; Figure 3B) into the MHD motif of APAF-1 30 (vanderBiezen and Jones, 1998) or similar animal proteins it is likely that there would be cell death.

Apoptosis may be assessed by those skilled in the art using commercial kits see e.g. Oncogene Research Products, 84 Rogers 35 St., Cambridge, MA 02142, (1999) General Catalog pp 21-55. The modified proteins used herein may be of particular interest for investigating regulators of apoptosis e.g. cellular initiators

(cf. elicitors) or inhibitors.

FIGURE LEGENDS

5 Figure 1: Induction of HR by an auto-activator mutant of Rx and expression of the auto-activator Rx from a PVX vector

A) Schematic representation of T-DNA constructs for expression of Rx cDNA.

10 The cDNA inserts of wild type or mutant forms of Rx were inserted between Rx promoter (pR) and transcriptional terminator (ter). The black box indicates the cDNA or either wild type (wt) Rx cDNA or the cDNA of mutant (AT) forms of Rx. LB and RB indicate the left and right border of the T-DNA.

15

B) Schematic representation of the T-DNA of the PVX vector expressing an auto-activator.

LB and RB are the left and right borders of the T-DNA and 35S and Nos indicate the promoter and nopaline synthase transcription 20 terminator. The PVX open reading frames are shown as grey boxes with 'CP' indicating the PVX coat protein; the three boxes labeled 'mv' indicate the PVX genes required in virus movement and replicase is the replication enzyme. The diagram is not to scale. In PVX-AT* the coat protein gene was replaced with the coding 25 sequence of auto-activators pR-AT25 (PVX-AT25), pR-AT39 (PVX-AT39) or by a deletion mutant of pR-AT25 (PVX-AT00).

Figure 2: Identification of autoactivating mutations.

30 Each of the auto-activator mutant forms of Rx had several coding sequence mutations. To identify the mutations responsible for the autoactivation of HR a series of Rx constructs was prepared in which wild type Rx and the auto-activator mutants were recombined using the restriction sites indicated at the top of the panel. The 35 constructs were transformed into agrobacterium and ability of these constructs to activate HR was hybrid clones were then tested by infiltration in non transformed (NT) and coat protein (CP)

transgenic tobacco. The ability to induce HR is indicated by '+' and '-' indicates that the construct was tested but that there was no HR. The thin line indicates the wild type Rx sequence and the thick line indicates sequence derived from the auto-activators 5 mutants. The numbers above the thick lines refer to the number of amino acids that vary between Rx and the auto-activator mutant.

Figure 3: The primary structure of the Rx auto-activator proteins

10 A) The primary structure of the Rx protein.

The predicted sequence of Rx has been divided into seven regions.

Region 1 contains the a leucine zipper -like region;

regions 2 and 3 contain the NB-ARC domain, which is the domain containing the conserved motifs shown in upper case letters

15 between 168 and 260 (the ARC domain is between 260 and 472); region 4 includes the leucine rich repeats (each LRR is shown on a different line)and regions 5, 6 and 7 are respectively rich in amide, basic and acidic residues as described previously (Bendahmane et al., 1999). The conserved NB-ARC domain residues 20 are shown in upper case bold and the residues responsible for the autoactivation mutants is are shown as white on a black background.

B) Amino acid substitutions that lead to autoactivation of Rx

25

The amino acids labelled in blocks in wild type Rx (left hand side) were changed, as indicated, in the auto-activators (right hand side). The position of the modifications in Rx can be determined by cross reference to panel A.

30

Figure 4: Autoactivation of HR by truncated and overexpressed Rx

A schematic representation of Rx constructs in which the promoters were 35S or from Rx (pR). The transcriptional terminators were 35 from the 35S transcript of CaMV(35 T) or Rx (Ter) and the constructs were based on the leucine zipper (LZ), NB-ARC (regions 1-3; Figure 3) and on the LRR (region 4; Figure 3) of Rx.

The black box represents the C terminal regions 5-7 (Figure 3) of Rx. The '+' or "--" indicates whether an HR was induced when the constructs were expressed using agrobacterium infiltration in the leaves of non transgenic tobacco.

5

Figure 5: Forced dimerization of Rx leads to HR.

A) Dim-Rx and Rx-Dim indicate constructs with an N terminal or C terminal fusion of the FKBP12 dimerizing domain (Dim) to Rx were 10 inserted into the expression cassette of pBIN61in which the transcription promoter (35S) and terminator (35T) were both from the 35S transcript of cauliflower mosaic virus. The constructs were based on the leucine zipper (LZ), NB-ARC (regions 1-3; Figure 3) and on the LRR (regions 4-7; Figure 3) of Rx.

15

B) The constructs were expressed in non transgenic (NT) or coat protein transgenic tobacco leaves by agrobacterium transient expression assay either in the presence or absence of the dimerization agent AP20187(AP). The '+' or "--" indicates whether 20 an HR was induced.

EXAMPLES

Example 1- Random mutagenesis of Rx

25

To address a possible role of the NB-ARC and other domains we carried out a mutation analysis of Rx in which the screen was for activation of a resistance response in the absence of the PVX coat protein. First Rx mutants were generated by PCR of Rx cDNA under 30 conditions leading to mis-incorporation of nucleotides and the PCR products were inserted between the promoter and transcriptional terminator of Rx (Figure 1A, pr-AT). The constructs were assembled in the T-DNA region of an agrobacterium binary plasmid vector and a library of mutants was generated by transformation of 35 agrobacterium. Individual clones were assayed for biological activity by infiltration of liquid agrobacterium cultures into leaves of tobacco.

When the agrobacterium cultures carrying a wild type Rx cDNA construct (pR-Rx; Figure 1A) were infiltrated into non transformed tobacco leaves there was no visible effect. However, when this construct was infiltrated into transgenic tobacco producing the 5 coat protein of PVX, there was necrosis after 48h and death of the infiltrated region by 72h. Presumably this hypersensitive response (HR) resulted from transient transformation of cells in the infiltrated region of the leaf. Rx would have recognised the coat protein and activated signalling leading to cell death.

10

Out of 2500 mutant Rx clones tested we identified seven that induced an HR in the leaves of non transformed tobacco. We refer to these mutants as auto-activators.

15 The HR phenotype was assessed using Rx constructs which were the wild type Rx (pR-Rx) or the auto-activator mutant derivative (pR-AT25). Agrobacterium carrying pR-Rx or pR-AT25 was infiltrated into leaves of either non transformed or transgenic tobacco expressing the PVX coat protein from the 35S promoter. The leaves 20 were photographed 4 days after infiltration.

Four of the auto-activator forms of Rx (pR-AT39, pR-AT193, pR-AT25, and pR-AT7) had the phenotype of the AT25 mutant shown in Figure 1B in which the HR was induced within 48h. The others 25 (pR-AT32, pR-AT72 and pR-AT28) induced an HR that was delayed until 48-72h post infiltration.

DNA sequence analysis revealed that each of the auto-activator forms of Rx carries between 3 and 11 amino acid substitutions 30 relative to the wild type. To identify those amino acid substitutions implicated in the autoactivation of Rx we constructed a series of recombinant molecules incorporating elements of the wild type and auto-activator Rx (Figure 2). These recombinant molecules were assayed for the ability to autoactivate 35 HR using the agrobacterium infiltration assay on non transformed tobacco plants.

The pR-AT39, pR-AT193, pR-AT25, pR-AT32, pR-AT72 and pR-AT28 clones all contained a single mutation that was responsible for HR activation (Figure 2). In each instance the timing of the HR was the same with the single amino acid mutant and the corresponding 5 progenitor clone. For example, the pR-AT39 and its derivative with a single amino acid change relative to wild type Rx both induced a rapid HR. In contrast the pR-AT7 mutant carried two mutations that were independently responsible for autoactivation of the HR (Figure 2). In both instances, and unlike the progenitor pR-AT7, 10 the single amino acid mutants induced a rapid HR. Presumably one or more of the other seven mutations in pR-AT7 impaired the ability of the encoded protein to activate the HR (Bendahmane et al., 1999).

15 The distribution of the autoactivating mutations is non- random (Figure 3A). Three out of eight of these mutations (pR-AT25, pR-AT32 and pR-AT72) were within a 6 amino acid interval close to the CFLY motif and a further two (pR-AT39 and pR-AT193) were in the MHD motif (Figure 3A and 3B). The two mutations in the MHD 20 motif were D to V substitutions, although with different changes at the nucleotide level. Both the MHD and CFLY motifs are components of the NB-ARC domain. The remaining three autoactivating mutations were in LRR2 (pR-AT7), LRR4 (pR-AT7) and LRR11 (pR-AT28) (Figure 3A and 3B).

25

We carried out two tests to confirm that the auto-activator mutations led to a response that was the same as the Rx mediated resistance in virus infected cells. First we introduced mutations into Rx that would interfere with the ability of Rx to activate 30 resistance in the presence of the PVX coat protein and tested whether these mutations also prevented the auto-activator induced HR. In the second test we assayed whether the auto-activators, like Rx, would confer virus resistance independently of an HR.

35 In the first of these tests the mutations were alanine substitutions in motifs I (pR-RxK1), III (pR-RxK2) and V (pR-RxGL) of the Ap-ATPase domain of the NB-ARC homologous region (Aravind

et al., 1999). The assay of the Rx response was based on the HR following the agrobacterium infiltration assay into PVX coat protein transgenic plants. In each instance the introduction of the mutation into the wild type Rx blocked the HR in the 5 infiltrated region of the coat protein transgenic plants.

The results are shown in Table 1. To compile the table, mutations were introduced into the motif I (K1), III (K2) and V (GL) of the Ap-ATPase domain of either the wild type Rx cDNA (pR-Rx) or the 10 cDNA of auto-activator mutants (pAT). The pAT39(6) construct was the derivative of pAT39 in which the only change from wild type Rx was a single D to V substitution in the MHD motif; the pAT25(33) construct was the derivative of pAT25 in which the only change from wild type Rx was the CFLY motif mutation; the pR-At7(30) 15 construct had the with the D to E and H to R substitutions from the LRR of pR-At7 as the only differences from wild type Rx cDNA. The constructs were expressed in non transgenic or coat protein transgenic tobacco leaves by agrobacterium transient expression assay.

20

The table indicates that the Ap-ATPase domains are essential for Rx function.

Similarly, when the mutations were introduced into the background 25 of the auto-activators, the HR was blocked. Thus the Ap-ATPase mutants of auto-activator pR-AT39(6) did not induce an HR in non transformed tobacco leaves. This mutation was in the MHD motif (D to V substitution). The Ap-ATPase mutations also blocked the HR in the background of pR-AT25(33) carrying the CFLY motif mutation of 30 pR-AT25 and in pR-AT7(30) with the D to E and H to R substitutions from the LRR of pR-AT7. From these results we conclude that the HR pathway induced by the auto-activators was similar to the HR pathway induced when Rx was elicited by PVX coat protein.

35 To assay for HR-independent virus resistance induced by the auto-activators we exploited our previous observation that there are two branches of the Rx response pathway. There is one response

leading to suppression of virus accumulation and a secondary response leading to the HR. We predicted that, if virus resistance was a response of the auto-activators, it could be assessed by use of PVX vector constructs. The constructs used for this test

5 (Figure 1B) had the CP ORF in PVX replaced with the AT39 and AT25 forms of Rx in which there were auto-activator mutations in the MHD and CFLY motifs of the NB-ARC domain respectively. The coat protein replacement in the control construct (AT00) encoded the AT-25 auto-activator with a deletion of the motif I from the
10 Ap-ATPase domain. We reasoned that if the PVX-AT39 or PVX-AT25 accumulated more slowly than PVX-AT00 it would indicate that the auto-activator response leads to suppression of virus accumulation.

15 These various PVX constructs were inserted under the control of a constitutive cauliflower mosaic virus 35S (35S) promoter in the expression cassette of an agrobacterium binary plasmid vector. The vectors were transformed into agrobacterium and inoculation was by infiltration of liquid cultures into tobacco leaves. RNA was
20 extracted from the infiltrated region after two days, when there was no evident cell death and northern blotting was used to assay viral RNA accumulation.

25 Specifically, agrobacterium carrying PVX-AT25, PVX-AT39 or PVX-AT00 were infiltrated into tobacco leaves. Two days after agroinfiltration and prior to the appearance of cell death, total RNA was extracted from the infiltrated patch and PVX accumulation was tested by RNA blot analysis. Each lane of the gel was loaded with 2 µg of total RNA. The hybridisation probe was a riboprobe
30 specific for the positive strand RNA of PVX (results not shown).

In the PVX-AT00 inoculated tissue there was high level accumulation of PVX genomic RNA and, indicative of virus replication, there were also subgenomic RNAs.

In contrast the genomic RNAs of PVX-AT39 and PVX-AT25 constructs were substantially less abundant than with PVX-AT00 and the

subgenomic RNAs were not detectable. Therefore, the mutant forms of Rx activated both the primary virus resistance and secondary HR components of the Rx response.

5 Example 2 - over-expression of Rx

The finding that five out of eight auto-activator mutations were clustered in conserved motifs of the NB-ARC domain prompted us to explore further the possible similarity of Rx with CED4 and 10 APAF-1. First we tested the possibility that overexpression would activate Rx-mediated HR in the way that overexpression CED4 leads to apoptosis in *C. elegans* (Shaham and Horvitz, 1996) and APAF-1 overexpression potentiates apoptosis in mammalian cells (Hu et al., 1998b).

15

To test the effects of Rx overexpression we modified the Rx constructs in agrobacterium binary plasmid vector so that the weak Rx promoter was replaced with the strong constitutive 35S promoter. Agrobacterium cultures containing these constructs were 20 then infiltrated, as described above, into non transformed or tobacco. With Rx under its own promoter there was no HR under these conditions. However, with the 35S construct, there was a strong HR. There was also an HR with a 35S construct encoding Rx with a C terminal deletion (35S-NBS; Figure 4). This deleted form 25 of Rx lacked the LRR domains but retained the NB-ARC domain in which there was similarity with CED-4 and APAF-1. There was no HR when this construct was expressed under control of the Rx promoter (pR-NBS; Figure 4) or when the C terminal part of Rx including the LRR was expressed from the 35S promoter (Figure 4).

30

These results indicate that the N terminal part of Rx, including the NB-ARC domain, has all of the information required for signalling of the response pathway. Moreover, by showing that the LRR is not required for coat protein-independent HR, these data 35 are consistent with the previous suggestions that the LRR is concerned with recognition rather than response.

Example 3 - dimerization of Rx

A key process in one of the pathways of animal cell apoptosis is the dimerization of CED4/APAF-1 (Hu et al., 1998a; Srinivasula et 5 al., 1998; Yang et al., 1998). This dimerization activates a caspase cascade leading ultimately to cell death. To find out whether dimerization of Rx regulates disease resistance in plants we used a system (Amara et al., 1997; Clackson et al., 1998) based 10 on a nontoxic lipid-permeable reagent, AP20187, that cross links the FKBP12 protein. The system is discussed more fully in the Experimental Procedures below. In the presence of AP20187 proteins carrying a FKBP12 are forced to dimerize. Rx constructs were made with either an N terminal or C terminal fusion of the FKBP12 and activation of an HR was tested by agrobacterium infiltration.

15

In initial tests these Rx constructs were under control of the Rx promoter and there was no HR in the presence of AP20187 or even when the constructs were assayed in transgenic plants expressing the PVX coat protein. The FKBP12 fusions had either destabilized 20 or inactivated Rx. Consistent with that idea, the fusion constructs were unlike the wild type Rx in that expression from the 35S promoter did not lead to an HR in the absence of PVX coat protein (Figures 4 and 5). However in the presence of the PVX coat protein there was an HR with both DimRx and RxDim expressed from 25 the 35S promoter. There was also an HR when the C terminal fusion (Rx-Dim; Figure 5) was assayed in the presence of the AP20187 dimerizer but not with the N terminal fusion (Dim-Rx; Figure 5). These data provide strong support for the notion that control of Rx-mediated resistance involves dimerization of the Rx protein.

30

EXPERIMENTAL PROCEDURES*Rx constructs*

35 pB1 is a modified pBIN19 plasmid (Bevan, 1984) that carries a transcription cassette comprising 3 kb of the Rx promoter and a 1.5 kb Rx terminator separated by an XbaI and a SacI cloning sites

(Bendahmane and Baulcombe, 1999). All Rx derivative mutants were cloned between the XbaI and the SacI cloning sites. To construct the pB1 binary vector, the Rx promoter was PCR-amplified using the primers RxP4 (TCG GGG TAC CTC TAT TGA AGA ATT GAG ATC CAA G) and RxP2 (CTC AGT ATC TAG ATG AAC AAA TTG CC) and the PCR product was digested with XbaI. The Rx terminator was also PCR-amplified using primers RxT1 (CAG CTG TAA GCT CGT TGA TAT AGA GG) and RxT2 (GGT GTT CTA GAG ACT AGC CAG AGC TCT GAA AT) and the PCR product was digested with XbaI and KpnI. In each instance the BAC77 DNA carrying the Rx1genomic DNA (Bendahmane et al., 1999) was used as template for the PCR. The digested PCR products were ligated to a modified pBIN19 plasmid vector digested with KpnI and EcoRI to create pB1. The modified pBIN19 plasmid is identical to the one published previously except that the unique XbaI site was deleted.

To construct pR-Rx, Rx cDNA was PCR amplified with the primers RxP1 (GGC AAT TTG TTC ATC TAG ATA CTG AGA GA) and Rxac4 (TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC). The PCR product was digested with XbaI and SacI and ligated to pB1 digested with XbaI and SacI to create pR-Rx.

The 35S promoter constructs were all in the pBIN61 binary vector. pBIN61 is a modified pBIN19 binary vector that carries a transcription cassette comprising the CaMV 35S promoter and 25 terminator. To construct the pBIN61 binary vector, the transcription cassette containing the CaMV 35S promoter and terminator was released by digestion with KpnI and XbaI from the plasmid pJIT61 (kindly provided by P. Mullineaux, JIC, Norwich, UK). The transcription cassette was then ligated to the pBIN19 plasmid vector digested with KpnI and SalI to create pBIN61.

To construct 35S-Rx, Rx cDNA was PCR amplified with primers RxP1 (GGC AAT TTG TTC ATC TAG ATA CTG AGA GA) and Rxac4 (TAT TTC AGA GCT CTG GCT AGT CCT CAG AAC ACC). The PCR product was digested 35 with XbaI and ligated with pBIN61 digested with XbaI and SmaI.

Truncated forms of Rx were constructed in pBIN61 using chimaeric

PCR as described previously (Ho et al., 1989). The primers were designed to allow PCR amplification of the 5' part of the Rx coding sequence encoding regions 1-3 (Figure 3) and the 3' part encoding regions 5-7 (Figure 3) in separate reactions. The second 5 stage of the chimaeric PCR was then used to fuse the two parts in frame with the LRR deleted (region 4; Figure 3). Primers used for the production of these truncated forms of Rx were:

RxP1:GGCAATTGTTCATCTAGATACTGAGAGA

Rxac4:TATTCAGAGCTCTGGCTAGTCCTCAGAACACC

10 LRR1:ATTCACGTGAGATTGTTGGTTCGAGCTTCCCTCAA

LRR2:CAACAATCTGTTGTGAATTCCGCC

The first stage PCR reactions were carried out with the primers RxP1 and LRR1 (PCR1) and LRR2 and Rxac4 (PCR2). In the second stage PCR the product of PCR1 was mixed with the product of the 15 PCR2 and PCR amplified with primers RxP1 and Rxac4.

To prepare the 35S-LRR construct the LRR domain was PCR amplified with the primers LRR/XbaI: (GAA GCT CTA GAC ATG AAT TTT GTG AAT) and ATSal: (AAC TGT CGA CTC CTC AGA ACA CCT T). The PCR product 20 was digested with XbaI and ligated to pBIN61 digested with XbaI and Ecl 136 to create 35S-LRR.

The N terminal (DimRx) and C terminal (RxDim) translation fusion between Rx cDNA and a tandem repeat of the dimerizing domain 25 FKBP12 were made by chimeric PCR (Ho et al., 1989), as described previously. Primers used to make the fusion between Rx and the dimerization domain were:

a) DimRx

DimF1: CCCATCTAGATGAGCAGAGGCGTCCAAGTC

30 DimF2: GAAACTAGTATGGCTTATGCTGCTGTT

DimR1: ATAAGCCATACTAGTTCCAGTTTAG

Rx8: AATTGGCCATGTATTCAAACCAAG

b) RxDim

35 DimF3: AATGTCGAGAGCAGAGGCGTCCAAGTCGAA

DimR2: GCCTCTGCTCTCGACATTATTGCGGGCA

DimR3: GTCAGAGCTTTATGCGTAGTCTGG

K29: TGGTTGGCCGTGAAAATGAA

The constructs were prepared in the Rx promoter cassette of pB1 and in the 35S cassette of pBin61.

5

PVX constructs

The open reading frames of auto-activators AT39 and AT25 were PCR amplified with the primers corresponding to the 5' and 3' extremes 10 of the Rx cDNA. The PCR products were digested with SalI and ligated to the PVX vector construct pgR108 digested with SmaI and XhoI. pRG108 is essentially the same as the previously described PVX vectors (Chapman et al., 1992) except that it is under control of the 35S promoter in the pGreen binary vector. A second 15 modification is that the insertion site of foreign sequence has been modified so that several restriction sites including SmaI can be used for insertion of sequences into the PVX vector.

The insertion of sequence between the SmaI and XhoI sites of 20 pgR108 resulted in replacement of most of the PVX coat protein coding sequence with the Rx auto-activator. The PVX clones that express the auto-activators AT-25 and AT-6 are referred to as PVX-AT25 and PVX-AT6, respectively. PVX-AT00 is the same as PVX-AT25 except for a deletion of the first 243 amino acids of the 25 protein containing the motif I of the Ap-ATPase domain.

PCR mutagenesis

Random mutagenesis of the Rx gene was performed under conditions 30 similar to those previously described (Shafikhani et al., 1997). The PCR was carried out using the primers RxP1 and Rxac4 which flank the Rx ORF. The PCR reaction contained (100 μ l final volume) 10 mM Tris (pH 8.3), 50 mM KCl, 0.05% Nonidet P-40, 7 mM MgCl₂, 0.15 mM MnCl₂, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 0.3 35 μ M of both primers, 50 ng of template and 5 U Taq DNA polymerase (GIBCO-BRL). PCR was performed for 35 cycles: 15 s at 94 °C, 15 s at 55 °C, and 2 min at 72 °C. The PCR products were digested with

XbaI and SacI, gel purified and cloned in the binary vector pB1 in *E. coli*. Plasmid DNA was purified from 10000 colonies and electroporated into *A. tumefaciens* strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996).

5

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis (Bendahmane et al., 1995) was used to introduce specific mutations into the Rx cDNA or into the 10 auto-activators AT25(33), AT39(6) pAT7(30). The presence of mutations was confirmed by sequence analysis. The mutations of the Ap-ATPase motif I were at Rx codon 175 from GGG(G) to GCG(A) and at codon 176 from AAA(K) to GCA(A). The mutations of the Ap-ATPase motif III were at Rx codon 244 from GAT(D) to GCT(A) and at codon 15 245 from GAC(D) to GCC(A). The mutations of the Ap-ATPase motif V were at codons 330 from GGA(G) to GCA(A) and 332 from CCT(P) to GCT(A). The mutations of the CFLY motif were at position 389 from TGT(C) to GCT(A) and at position 390 from TTT(F) to GCT(A). The mutations of the MHD motif were at position 175 from GGG(G) to 20 GCG(A) and at position 176 from AAA(K) to GCA(A).

Agrobacterium-mediated transient expression

Agrobacterium-mediated transient expression was performed under 25 conditions similar to those described previously (Bendahmane et al., 1999). The binary Ti-plasmid vector constructs were transformed into *A. tumefaciens* strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996). The transformants were inoculated into 5 ml L-broth medium 30 supplemented with 50 µg/ml kanamycin and 5 µg/ml tetracycline and grown at 28°C overnight. Cells were precipitated and resuspended to the OD of 0.5 in solution containing 10 mM MgCl₂, 10 mM MES pH 5.6 and 150 µM acetosyringone. The cells were left at room temperature on the bench for 2 h before infiltration into tobacco leaves. The 35 infiltrations were either into non transformed tobacco or into transgenic tobacco expressing the PVX coat protein (Spillane et al., 1997).

Forced dimerization of Rx

The RxDim and DimRx constructs were assayed by agrobacterium infiltration, as described above but, as indicated in the text and 5 Figure 5, with the addition of the dimerization agent AP20187 (5 μ M final concentration) (Amara et al., 1997; Clackson et al., 1998) (ARIAD Pharmaceuticals, Inc. 26 Landsdowne Street Cambridge, MA 02139) immediately before infiltration into tobacco leaves.

10 *DNA sequencing and analysis*

The sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer). Sequence reactions were resolved on ABI377 automated sequencer (Applied Biosystems 15 ABI, La Jolla, CA). Sequence contigs were assembled using UNIX versions of the Staden programs package (Staden, 1996).

The Dimerizer system

20 The original dimerizer used by the Crabtree and Schreiber laboratories to create the model system was FK1012, which is composed of two molecules of the immunosuppressant drug FK506 covalently joined by a flexible linker. FK1012 efficiently dimerizes proteins fused to its cellular receptor FKBP12. FK1012 25 has been used successfully to regulate receptor activity, to change the intracellular localization of proteins, and to control gene expression (3, 4, 7, 9, 10).

A related molecule, FKCsA, is composed of one molecule of FK506 30 linked to a molecule of a distinct immunosuppressant drug, cyclosporin A (CsA) (6). FKCsA will dimerize an FKBP12-fusion protein to a second protein fused to cyclophilin A, the cellular receptor for CsA. FKCsA therefore selectively promotes the formation of heterodimers. The use of a heterodimerizer has 35 potential advantages in situations where the two proteins to be joined are different, such as in transcriptional regulation. In ARIAD's experience, however, the quantitative improvement over

simple homodimerizers is relatively small.

ARIAD's internal efforts also include the development of a gene regulation system for use in human gene therapy, one aspect of 5 which is built around a third immunosuppressant drug, rapamycin (8). Rapamycin efficiently links an FKBP12-fusion protein to a second protein fused to a domain of human FRAP, the target of the rapamycin/FKBP12 complex. Rapamycin itself is not optimal for use in human gene therapy because of its immunosuppressive activity. 10 Therefore, ARIAD is developing nonimmunosuppressive derivatives of rapamycin for its human gene therapy program.

For distribution to the academic community, ARIAD has synthesized a novel, proprietary dimerizer, AP1510. This molecule acts in a 15 manner similar to FK1012 in that it promotes the formation of FKBP12 homodimers. ARIAD is distributing this molecule for several reasons:

It is a versatile, multi-purpose dimerizer, effective in both 20 receptor dimerization and gene regulation applications. In ARIAD's experience

AP1510 works significantly better than FK1012 in both applications.

25 Unlike rapamycin, AP1510 is completely nontoxic to cells.

The other dimerizer molecules are composed of natural product compounds obtained by fermentation of microorganisms. In contrast, 30 AP1510 is entirely synthetic and is made in bulk by ARIAD chemists. In addition, ARIAD chemists continue to work toward building dimerizers of this class with improved properties.

35 Inasmuch as any of the following may be required to enable the performance of the invention, the reference is specifically

incorporated herein by cross-reference.

References for dimerizer discussion:

5 1. Austin et al. Proximity versus allosteric: the role of regulated protein dimerization in biology. *Chemistry & Biology* 1, 131-136 (1994).

10 2. Spencer, D.M. Creating conditional mutants in mammals. *Trends Genet.* 12, 181-187 (1996).

3. Spencer et al. Controlling signal transduction with synthetic ligands. *Science* 262, 1019-1024 (1993).

15 4. Prusky, M.N., et al. Mechanistic studies of a signaling pathway activated by the organic dimerizer FK1012. *Chemistry & Biology* 1, 163-172 (1994).

20 5. Spencer et al. Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. *Current Biology* 6, 839-847.

25 6. Belshaw et al. Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. *Proc. Natl. Acad. Sci. USA* 93, 4604-4607 (1996).

30 7. Ho et al. Dimeric ligands define a role for transcriptional activation domains in reinitiation. *Nature* 382, in press (1996).

8. Rivera et al. A humanized system for pharmacologic control of gene expression. *Nature Medicine* 2, 1028-1032 (1996).

35 9. Spencer et al. A general strategy for producing conditional alleles of Src-like tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 92, 9805-9809 (1995).

10. Holsinger et al. Signal transduction in T lymphocytes using a conditional allele of Sos. Proc. Natl. Acad. Sci. USA 92, 9810-9814 (1995).

5 Additional References

Amara et al (1997). A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc. Natl. Acad. Sci. USA 94, 10618-10623.

10

Angell, S. M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO J. 16, 3675-3684.

15 Aoyama, T., and Chua, N.-H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. 11, 605-612.

20 Aravind et al (1999). The domains of death: evolution of the apoptosis machinery. Trends in Biochemical Sciences 24, 47-53.

25 Bendahmane, A., and Baulcombe, D. C. (1999). Agrobacterium transient expression system as a tool for isolation of disease resistance genes: application to the Rx2 locus in potato. Plant J. submitted.

Bendahmane et al (1999). The Rx gene from potato controls separate virus resistance and cell death responses. Plant Cell 11, 781-791.

30 Bendahmane et al (1995). The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. Plant J. 8, 933-941.

35 Bevan, M. W. (1984). Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res. 12, 8711-8721.

Chapman et al (1992). Potato virus X as a vector for gene

expression in plants. *Plant J.* 2, 549-557.

Clackson et al (1998). Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity.

5 *Proc.Natl.Acad.Sci.USA* 95, 10437-10442.

Dixon et al (1996) *Cell* 84, 451-459.

Ellis et al (1999). Identification of regions in alleles of the
10 flax rust resistance gene L that determine differences in
gene-for-gene specificity. *Plant Cell* 11, 495-506.

Gilbert et al (1998). Elicitation of Rx-mediated resistance to PVX
in potato does not require new RNA synthesis and may involve a
15 latent hypersensitive response. *Mol.Plant-Microbe Interact.* 8,
833-835.

Grant et al (1995) *Science* 269, 843-846.

20 Hamilton et al (1996). Stable transfer of intact high molecular
weight DNA into plant chromosomes. *Proc. Natl. Acad. Sci. USA* 93,
9975-9979.

Hammond-Kosack, K. E., and Jones, J. D. G. (1997). Plant disease
25 resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48,
575-607.

Ho et al (1989). Site-directed mutagenesis by overlap extension
using the PCR. *Gene* 77, 51-59.

30 Hu et al (1998a). WD-40 repeat region regulates Apaf-1
self-association and procaspase-9 activation. *J. Biol. Chem.* 273,
33489-33494.

35 Hu et al (1998b). Bcl-X-L interacts with Apaf-1 and inhibits
Apaf-1-dependent caspase-9 activation. *Proc.Natl.Acad.Sci.USA* 95,
4386-4391.

Jones and Jones (1997) *Adv. Bot. Res.* 24, 89-167.

Jones et al (1994) *Science* 266, 789-793.

5 Meyers et al (1998). Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. *Plant Cell* 10, 1833-1846.

10 Pozo, O., and Lam, E. (1998). Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Current Biology* 8, 1129-1132.

15 Rouppe van der Voort et al (1999). Tight physical linkage of the nematode resistance gene Gpa2 and the virus resistance gene Rx on a single segment introgressed from the wild species *Solanum tuberosum* subsp. *andigena* CPC 1673 into cultivated potato. *Mol Plant Microbe Interact* 12, 197-206.

Salmeron et al (1996) *Cell* 86, 123-133.

20 Shafikhani et al (1997). Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization. *Biotechniques* 23, 304-310.

25 Shaham, S., and Horvitz, H. R. (1996). Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. *Genes & Development* 10, 578-591.

30 Spillane et al (1997). Concurrent Suppression of Virus Replication and Rescue of Movement-Defective Virus in Transgenic Plants Expressing the Coat Protein of Potato Virus X. *Virology* 236, pp.76-84.

35 Srinivasula et al (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Molecular Cell* 1, 949-957.

Staden, R. (1996). The Staden sequence analysis package. *Molecular*

Biotechnology 5, 233-241.

Staskawicz et al (1995). Molecular genetics of plant disease resistance. Science 268, 661-667.

5

Thomas et al (1997) *The Plant Cell* 9, 2209-2224.

van der Biezen, E. A., and Jones, J. D. G. (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene 10 products and regulators of cell death in animals. *Curr. Biol.* 8, 226-227.

vanderBiezen, E. A., and Jones, J. D. G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends 15 In *Biochemical Sciences* 23, 454-456.

Whitham et al (1994) *Cell* 78, 1101-1115.

Yang et al (1998). Essential role of CED-4 oligomerization in 20 CED-3 activation and apoptosis. *Science* 281, 1355-1357.

Table 1 - The effect of AP-ATPase mutations on wild type and auto-activator forms of Rx

Construct	Response on non transformed tobacco	Response on coat transgenic tobacco
5 protein		
pR-Rx	-	+
pR-RxK1	-	-
pR-RxK2	-	-
10 pR-RxGL	-	-
PAT25 (33)	+	nt
PAT25 (33)-K1	-	nt
PAT25 (33)-tK2	-	nt
15 PAT25 (33)-GL	-	nt
PAT39 (6)	+	nt
PAT39 (6)-K1	-	nt
PAT39 (6)-K2	-	nt
20 PAT39 (6)-GL	-	nt
pAT7 (3)	+	nt
pAT7 (30)-K1	-	nt
pAT7 (30)-K2	-	nt
25 pAT (30)-GL	-	nt

The '+' or "-" indicates whether an HR was induced and 'nt' indicates not tested.

Table II - the DNA sequence of auto-activator Rx clones

The DNA sequences are of the auto-activators 193, 25 32 39, 7 and 72 as indicated. The start and stop codons are underlined in the sequence of

5 auto-activator 193

193_Create
25_Created
32_Created
10 39_Reverse
7_Reverse TAAATTCTATA AATCTATTGT ATGTAAGAAA CATACTTATA TTTCATGAATA
72_Created

15 193_Create
25_Created
32_Created
39_Reverse
7_Reverse GATATGTGTA GGGTCTAATA ATGAATTATC CCAATTTCCTT CTACTTTTC
20 72_Created

193_Create
25_Created
25 32_Created
39_Reverse
7_Reverse CTGTCAGAGT CCTGCTTTTT CTTTTTCTTT TTCTTTTTA ACTTTGGTCT
72_Created

30
193_Create
25_Created
32_Created
39_Reverse
35 7_Reverse CTGCTTTGT CTACATGATG ATAAGGTTGG TGGACCTAGC TGGAAATGTG
72_Created

193_Create
40 25_Created
32_Created

39_Reverse
 7_Reverse ATGGAAATAG CTAGTAAAAG AAAGACCTTT GCATTTCTG TTTTCTTAAA
 72_Created

 5
 193_Create TCTA GATACTGAGA
 25_Created ACTA GATACTGAGA
 32_Created TCTA GATACTGAGA
 39_Reverse TCTA GATACTGAGA
 10 7_Reverse AACTGAAAAA TTACATAACT TGTGGCAATT TGTCATCTA GACACTGAGA
 72_Created TCGACTCTA GATACTGAGA

 193_Create GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA
 15 25_Created GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA
 32_Created GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA
 39_Reverse GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA
 7_Reverse GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA
 72_Created GATATTCTA TTTTTGGAT ATATGGCTTA TGCTGCTGTT ACTTCCCTTA

 20

 193_Create TGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG
 25_Created AGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG
 32_Created TGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG
 25 39_Reverse TGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG
 7_Reverse TGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG
 72_Created TGAGAACCAT ACATCAATCA ATGGAACCTTA CTGGATGTGA TTTGCAACCG

 30 193_Create TTTTATGAAA AGCTCAAATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA
 25_Created TTTTATGAAA AGCTCAAATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA
 32_Created ATTTATGAAA AGCTCAGATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA
 39_Reverse TTTTATGAAA AGCTCAAATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA
 7_Reverse TTTTATGAAA AGCTCAAATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA
 35 72_Created TTTTATGAAA AGCTCAAATC TTTGAGAGCT ATTCTGGAGA AATCCTGCAA

 193_Create TATAATGGGC GATCATGAGG GGTTAACAAAT CTTGGAAGTT GAAATCGTAG
 25_Created TATAATGGGC GATCATGAGG GGTTAACAAAT CTTGGAAGTT GAAATCGTAG
 40 32_Created TATGATGGGC GATCATGAGG GGTTAACAAAT CTTGGAAGTT GAAATCGTAG
 39_Reverse TATAATGGGC GATCATGAGG GGTTAACAAAT CTTGGAAGTT GAAATCGTAG

7	_Reverse	TATAATGGGC GATCATGAGG GGTAAACAAT CTTGGAAGTT GAAATCGTAG
72	_Created	TATAATGGGC GATCATGAGG GGTAAACAAT CTTGGAAGTT GAAATCGTAG
5	193_Create	AGGTAGCATA CACAACAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
25	_Created	AGGTAGCATA CACAACAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
32	_Created	AGGTAGCATA CACAGCAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
39	_Reverse	AGGTAGCATA CACAACAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
7	_Reverse	AGGTAGCATA CACAACAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
10	72_Created	AGGTAGCATA CACAACAGAA GATATGGTTG ACTCGGAATC AAGAAATGTT
15	193_Create	TTTTTAGCAC AGAATTGGA GGAAAGAACG AGGGCTATGT GGGAGATTT
25	_Created	TTTTTAGCAC AGAATTGGA GGAAAGAACG AGGGCTATGT GGGAGATTT
32	_Created	TTTTTAGCAC AGAATTGGA GGTAAGAACG AGGGCTATGT GGGAGATTT
39	_Reverse	TTTTTAGCAC AGAATTGGA GGAAAGAACG AGGGCTATGT GGGAGATTT
7	_Reverse	TTTTTAGCAC AGAATTGGA GGAAAGAACG AGGGCTATGT GGGAGATTT
72	_Created	TTTTTAGCAC AGAATTGGA GGAAAGAACG AGGGCTATGT GGGAGATTT
20	193_Create	TTTCGTCTG GAACAAGCAC TAGAATGCAT TGATTCCACC GTGAAACAGT
25	_Created	TTTCGTCTG GAACAAGCAC TAGAATGCAT TGATTCCACC GTGAAACAGT
32	_Created	TTTCGTCTG GAACAAGCAC TAGAATGCAT TGATTCCACC GTGAAACAGT
39	_Reverse	TTTCGTCTG GAACAAGCAC TAGAAAGCAT TGATTCCACC GTGAAACAGT
25	7_Reverse	TTTCGTCTG GAACAAGCAC TAGAATGCAT TGATTCCACC GTGAAACAGT
	72_Created	TTTCGTCTG GAACAAGCAC TAGAATGCAT TGATTCCACC GTGAAACAGT
30	193_Create	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAAACCACA AACTAGCTCG
25	_Created	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAAACCACA AACTAGCTCG
32	_Created	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAAACCACG AACTAGCTCG
39	_Reverse	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAAACCACA AACTAGCTCG
7	_Reverse	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAATCCACA AACTAGCTCG
72	_Created	GGATGGCAAC ATCGGACAGC ATGAAAGATC TAAAACCACA AACTAGCTCG
35	193_Create	CTTGTCAAGTT TACCTGAACA TGATGTTGAG CAGCCCCGAGA ATATAATGGT
25	_Created	CTTGTCAAGTT TACCTGAACA TGATGTTGAG CAGCCCCGAGA ATATAATGGT
32	_Created	CTTGTCAAGTT TACCTGAACA TGATGTTGAG CAGCCCCGGAGA ATATAATGGT
40	39_Reverse	CTTGTCAAGTT TACCTGAACA TGATGTTGAG CAGCCCCGAGA ATATAATGGT
7	Reverse	CTTGTCAAGTT TACCTGAACA TGATGTTGAG CAGCCCCGAGA ATATAATGGT

72_Created CTTGTCAGTT TACCTGAACA TGATGTTGAG CAGCCCGAGA ATATAATGGT

193_Create TGGCCGTGAA AATGAAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG
5 25_Created TGGCCGTGAA AATGAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG
32_Created TGGCCGTGAA AATGAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG
39_Reverse TGGCCGTGAA AATGAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG
7_Reverse TGGCCGTGAA AATGAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG
10 72_Created TGGCCGTGAA AATGAATTG AGATGATGCT GGATCAACTT GCTAGAGGAG

193_Create GAAGGGAACT AGAACGTTGTC TCAATCGTAG GGATGGGAGG CATCGGGAAA
25_Created GAAGGGAACT AGAACGTTGTC TCAATCGTAG GGATGGGAGG CATCGGGAAA
32_Created GAAGGGAACT AGAACGTTGTC TCAATCGTAG GGATGGGAGG CATCGGGAAA
15 39_Reverse GAAGGGAACT AGAACGTTGCC TCAATCGTAG GGATGGGAGG CATCGGGAAA
7_Reverse GAAGGGACCT AGAACGTTGTC TCAATCGTAG GGATGGGAGG CATCGGGAAA
72_Created GAAGGGAACT AGAACGTTGTC TCAATCGTAG GGATGGGAGG CATCGGGAAA

20 193_Create ACAACTTGG CTACAAAACT CTATAGCGAT CCGTGCATTA TGTCTCGATT
25_Created ACAACTTGG CTACAAAACT CTATAGTGAT CCGTGCATTA TGTCTCGATT
32_Created ACAACTTGG CTACAAAACT CTATAGTGAT CCGTGCATTA TGTCTCGATT
39_Reverse ACAACTTGG CTACAAAACT CTATAGTGAT CCGTGCATTA TGTCTCGATT
7_Reverse CCACCTTGG CTACAAAACT CTATAGTGAT CCGTGCATTA TGTCTCGATT
25 72_Created ACAACTTGG CTACAAAACT CTATAGTGAT CCGTGCATTA TGTCTCGATT

193_Create TGATATTCGT GCAAAAGCAA CTGTTTCACA AGAGTATTGT GTGAGAAATG
25_Created TGATATTCGT GCAAAAGCAA CTGTTTCACA AGAGTATTGT GTGAGAAATG
30 32_Created TGATATTCGT GCAAAAGCAA CTGTTTCACA AGAGTATTGT GTGAGAAATG
39_Reverse TGATATTCGT GCAAAAGCAA CTGTTTCACA AGAGTATTGT GTGAGAAATG
7_Reverse TGATATTCGT GCAAAAGCAA CTGTTTCCCA AGAGTATTGT GTGAGAAATG
72_Created TGATATTCGT GCAAAAGCAA CTGTTTCACA AGAGTATTGT GTGAGAAATG

35 193_Create TACTCCTAGG CCTTCTTCT TTGACAAGTG ATGAACCTGA TGATCAGCTA
25_Created TACTCCTAGG CCTTCTTCT TTGACAAGTG ATGAACCTGA TGATCAGCTA
32_Created TACTCCTAGG CCTTCTTCT TTGACAAGTG ATGAACCTGA TGATCAGCTA
39_Reverse TACTCCTAGG CCTTCTTCT TTGACAAGTG ATGAACCTGA TGATCAGCTA
40 7_Reverse TACTCCTAGG CCTTCTTCT TTGCCAAGTG ATGACCCTGA TGATCAGCTA
72_Created TACTCCTAGG CCTTCTTCT TTGACAAGTG ATGGACCTGA TGATCAGCTA

193_Create	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
25_Created	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
32_Created	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
5 39_Reverse	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
7_Reverse	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
72_Created	GC GGACCGAC TGCAAAAGCA TCTGAAAGGC AGGAGATACT TGGTAGTCAT
10 193_Create	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
25_Created	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
32_Created	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
39_Reverse	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
7_Reverse	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
15 72_Created	TGATGACATA TGGACTACAG AAGCTTGGGA TGATATAAAA CTATGTTCC
193_Create	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
25_Created	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
20 32_Created	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
39_Reverse	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
7_Reverse	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
72_Created	CAGACTGTTA TAATGGAAGC AGAATACTCC TGACTACTCG GAATGTGGAA
25	
193_Create	GTGGCTGAAT ATGCTAGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
25_Created	GTGGCTGAAT ATGCTGGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
32_Created	GTGGCTGAAT ATGCTAGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
39_Reverse	GTGGCTGAAT ATGCTAGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
30 7_Reverse	GTGGCTGAAT ATGCTAGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
72_Created	GTGGCTGAAT ATGCTAGTTC AGGTAAGCCT CCTCATCACA TGCGCCTCAT
193_Create	GTATTTGAC GAAAGTTGGA ATTTACTACA CAAAAAGATC TTTGAAAAAG
25_Created	GAATTTGAC GAAAGTTGGA ATTTACTACA CAAAAAGATC TTTGAAAAAG
32_Created	GAATTTGAC GAAAGTTGGA ATTTACTACA CAAAAAGATC TTTGAAAAAG
39_Reverse	GAATTTGAC GAAAGTTGGA ATTTACTACA CAAAAAGATC TTTGAAAAAG
7_Reverse	GAATTTGAC GAAAGTTGGA ATTTACTACA CAAAAGGATC TTTGAAAAAG
72_Created	GAATTTGAC GAAAGTTGGA ATTTACTACA CAAAAAGATC TTTGAAAAAG

193_Create	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA
25_Created	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA
32_Created	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA
39_Reverse	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA
5 7_Reverse	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA
72_Created	AAGGTTCTTA TTCTCCTGAA TTTGAAAATA TTGGGAAACA AATTGCATTA

193_Create	AAATGTGGAG	GATTACCTCT	AGCAATTACT	GTGATTGCTG	GACTTCTCTC
10 25_Created	AAATGTGGAG	GATTACCTCT	AGCAATTACT	GTGATTGCTG	GACTTCTCTC
32_Created	AAATGTGGAG	GATTACCTCT	AGCAATTACT	GTGATTGCTG	GACTTCTCTC
39_Reverse	AAATGTGGAG	GATTACCTCT	AGCAATTACT	GTGATTGCTG	GACTTCTCTC
7_Reverse	AAATGTGGAG	GATCACCTCT	AGCAATTACT	GTGATTGCTG	GACTCCTCTC
72_Created	AAATGTGGAG	GATTACCTCT	AGCAATTACT	GTGATTGCTG	GACTTCTCTC

15

193_Create	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA
25_Created	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA
32_Created	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA
20 39_Reverse	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA
7_Reverse	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA
72_Created	CAAAATGGGT CAAAGATTAG ATGAGTGGCA AAGAATTGGG GAAAATGTAA

```

25 193_Create      GTTCGGTCGT TAGCACAGAT CCTGAAGCAC AATGCATGAG AGTGTGGAT
    25_Created      GTTCGGTCGT TAGCACAGAT CCTGAAGCAC AATGCATGAG AGTGTGGCT
    32_Created      GTTCGGTCGT TAGCACAGAT CCTGAAGCAC AATGCATGAG AGTGTGGCT
    39_Reverse      GTTCGGTCGT TAGCACAGAT CCTGAAGCAC AATGCATGAG AGTGTGGCT
    7_Reverse       GTTCGGTCGT TAGCACAGAT CCTGAAGCCC AATGCATGAG AGTGTGGCT
30 72_Created      GTTCGGTCGT TAGCACAGAT CCTGAAGCAC AATGCATGAG AGTGTGGCT

```

	193_Create	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATTT
	25_Created	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATTT
35	32_Created	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATAT
	39_Reverse	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATTT
	7_Reverse	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATTT
	72_Created	TTGAGTTACC ATCACTTGCC TTCTCACCTA AAACCGTGT TTCTGTATTT

40

193 Create TGCAACTTTC ACAGAGGATG AACAGATTTC TGTAATGAA CTTGTTGAGT

25_Created TGCAATTTTC ACAGAGGATA AACAGATTTC TGTAAATGAA CTTGTTGAGT
 32_Created TGCAATTTTC ACAGAGGATG AACAGATTTC TGTAAATGAA CTTGTTGAGT
 39_Reverse TGCAATTTTC ACAGAGGATG AACAGATTTC TGTAAATGAA CTTGTTGAGT
 7_Reverse TGCAATTTTC ACAGAGGATG AACAGATTTC TGTAAATGAA CTTGTTGAGT
 5 72_Created TGCAATTTTC ACAGAGGTTG AACAGATTTC TGTAAATGAA CTTGTTGAGT

193_Create TATGGCCTGT AGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA
 25_Created TATGGCCTGT AGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA
 10 32_Created TATGGCCTGT GGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA
 39_Reverse TATGGCCTGT AGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA
 7_Reverse TATGGCCTGT AGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA
 72_Created TATGGCCTGT AGAGGGATT TTGAATGAAG AAGAGGGAAA AAGCATAGAA

15 193_Create GAGGTGGCAA CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT
 25_Created GAGGTGGCAA CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT
 32_Created GAGGTGGCAA CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT
 39_Reverse GAGGTGGCAA CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT
 20 7_Reverse GAGGTGGCAC CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT
 72_Created GAGGTGGCAA CAACATGTAT AAACGAACCTT ATAGATAGAA GCTTAATT

193_Create CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 25 25_Created CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 32_Created CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 39_Reverse CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 7_Reverse CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 72_Created CATCCACAAT TTTAGTTTTC GTGGAACAAT AGAAAGTTGT GGAATGCATG
 30

193_Create TTGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG
 25_Created ATGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG
 32_Created ATGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG
 35 39_Reverse TTGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG
 7_Reverse ATGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG
 72_Created ATGTGACCCG TGAACTCTGT TTGAGGGAAG CTCGAAACAT GAATTTGTG

40 193_Create AATGTTATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA
 25_Created AATGTTATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA

32_Created AATGTTATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA
 39_Reverse AATGTTATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA
 7_Reverse AATGTCATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA
 72_Created AATGTTATCA GAGGAAAGAG TGATCAAAAT TCATGTGCAC AATCCATGCA
 5

193_Create GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT
 25_Created GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT
 32_Created GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT
 10 39_Reverse GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT
 7_Reverse GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT
 72_Created GCGTTCCCTT AAGAGTCGAA GTCGGATCAG AATCCATAAG GTGGAAGAAT

15 193_Create TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA
 25_Created TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA
 32_Created TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA
 39_Reverse TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA
 7_Reverse TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA
 20 72_Created TGGCTTGGTG TCGTAACAGT GAGGCTCATT CTATTATCAT GTTGGGTGGA

193_Create TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 25_Created TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 25 32_Created TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 39_Reverse TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 7_Reverse TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 72_Created TTCAATGCG TCACACTGGA ATTGTCTTTC AAGCTAGTAA GAGTACTAGA
 30

193_Create TCTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA
 25_Created TCTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA
 32_Created TCTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA
 39_Reverse TCTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA
 35 7_Reverse ACTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA
 72_Created TCTTGGTTG AATACATGGC CAATTTTCC CAGTGGAGTA CTTTCTCTAA

193_Create TTCATTTGAG ATACCTATCT TTGCGTTTA ATCCTTGCTT ACAGCAGTAT
 25_Created TTCATTTGAG ATACCTATCT TTGCGTTTA ATCCTTGCTT ACAGCAGTAT
 40 32_Created TTCATTTGAG ATACCTATCT TTGCGTTTA ACCCTTGCTT ACAGCAGTAT

39_Reverse	TTCATTTGAG ATACCTATCT TTGCGTTTA ATCCTTGCAT ACAGCAGTAT
7_Reverse	TTCATTTGAG ATACCTATCT TTGCGTTTA ATCCTTGCCT ACAGCAGTAT
72_Created	TTCATTTGAG ATACCTATCT TTGCGTTTA ATCCTTGCCT ACAGCAGTAT
5	
193_Create	CAAGGATCGA AAGAAGCTGT TCCCTCATCA ATAATAGACA TTCCTCTATC
25_Created	CAAGGATCGA AAGAAGCTGT TCCCTCATCA ATAATAGACA TTCCTCTAAC
32_Created	CAAGGATCGA AAGAAGCTGT TCCCTCATCA ATAATAGACA TTCCTCTATC
39_Reverse	CAAGGATCGA AAGAAGCTGT TCCCTCATCA ATAATAGACA TTCCTCTATC
10 7_Reverse	CAAGGATCGA AAGAAGCTGT .CCCTCATCA ATAATAGACA TTCCTCTATC
72_Created	CAAGGATCGA AAGAAGCTGT TCCCTCATCA ATAATAGACA TTCCTCTATC
193_Create	
15 25_Created	GATATCAAGC CTATGCTATC TGCAAACCTTT TAAACTTAAC CTTCCATTTC
32_Created	GATATCAAGC CTATGCTATC TGCAAACCTTT TAAACTTAAC CTTCCATTTC
39_Reverse	GATATCAAGC CTATGCTATC TGCAAACCTTT TAAACTTAAC CTTCCATTTC
7_Reverse	GATATCAAGC CTATGCTATC TGCAAACCTTT TAAACTTAAC CTTCCATTTC
20 72_Created	GATATCAAGC CTATGCTATC TGCAAACCTTT TAAACTTAAC CTTCCATTTC
193_Create	CCAGTTATTA TCCTTCATA TTACCATCGG AAATTTGAC GATGCCACAA
25_Created	CCAGTTATTA TCCTTCATA TTACCGTCGG AAATTTGAC GATGCCACAA
32_Created	CCAGTTATTA TCCTTCATA TTACCATCGG AAATTTGAC GATGCCACAA
25 39_Reverse	CTAGTTATTA TCCTTCATA TTACCATCGG AAATTTGAC GATGCCGCAA
7_Reverse	CCAGTTATTA TCCTTCATA TTACCATCGG AAATTTGAC GATGCCACAA
72_Created	CCAGTTATTA TCCTTCATA TTACCATCGG AAATTTGAC GATGCCACAA
30 193_Create	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
25_Created	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
32_Created	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
39_Reverse	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
7_Reverse	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
35 72_Created	TTGAGGACGC TGTGTATGGG CTGGAATTAC TTGCGGAGTC ATGAGCCTAC
193_Create	AGAGAACAGA TTGGTTTGA AAAATTGCA ATGCCTCAAT CAATTGAACC
25_Created	AGAGAACAGA ATGGTTTGA AAAATTGCA ATGCCTCAAT CAATTGAACC
40 32_Created	AGAGAACAGA TTGGTTTGA AAAATTGCA ATGCCTCAAT CAATTGAACC
39_Reverse	AGAGAACAGA TTGGTTTGA AAAATTGCA ATGCCTCAAT CAATTGAACC

7_Reverse AGAGAACAGA TTGGTTTGAA AAAATTGCA ATGCCTCAAT CAATTGAACC
 72_Created AGGGAACAGA TTGGTTTGAA AAAATTGCA ATGCCTCAAT CAATTGAACC

5 193_Create CTCGGTATTG TACAGGGTCT TTTTTAGAC TATTTCCAA TTTAAAGAAG
 25_Created CTCGGTATTG TACAGGGTCT TTTTTAGAC TATTTCCAA TTTAAAGAAG
 32_Created CTCGGCATTG TACAGGGTCT TTTTTAGAC TATTTCCAA TTTAAAGAAG
 39_Reverse CTCGGTATTG TACAGGGTCT TTTTTAGAC TATTTCCAA TTTAAAGAAG
 7_Reverse CTCGGTATTG TACAGGGTCT TTTTTAGAC TACTTCCAA TTTAAAGAAG
 10 72_Created CTCGGTATTG TACAGGGTCT TTTTTAGAC TATTTCCAA TTTAAAGAAG

15 193_Create TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA
 25_Created TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA
 32_Created TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA
 39_Reverse TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA
 7_Reverse TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA
 72_Created TTGCAAGTAT TTGGCGTCCC AGAAGACTTT CGCAATAGCC AGGACCTGTA

20 193_Create TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT
 25_Created TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT
 32_Created TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT
 39_Reverse TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT
 25 7_Reverse TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT
 72_Created TGATTTTCGC TACTTATATC AGCTCGAAGA ATTGACATTT CGTTTATATT

30 193_Create ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG
 25_Created ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG
 32_Created ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG
 39_Reverse ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG
 7_Reverse ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG
 72_Created ATCCATATGC TGCTTGCTTT CTAAAAAAACA CTGCACCTTC AGGTTCTACG

35 193_Create CAAGATCCTC TGAGGTTTCA GACGGAAATA TTGCACAAAG AGATTGATTT
 25_Created CAAGATCCTC TGAGGTTTCA GACGGAAATA TTGCACAAAG AGATTGATTT
 32_Created CAAGATCCTC TGAGGTTTCA GACGGAAATA TTGCACAAAG AGATTGATTT
 40 39_Reverse CAAGATCCTC TGAGGTTTCA GACGGAAATA TTGCACAAAG AGATTGATTT
 7_Reverse CAAGATCCTC TGAGGTTTCA GACGGAAATA TTGCACAAAG AGATTGATTT

72_Created CAAGATCCTC TGAGGTTCA GACGGAAATA TTGCACAAAG AGATTGATT

193_Create CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC
 5 25_Created CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC
 32_Created CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC
 39_Reverse CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC
 7_Reverse CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC
 10 72_Created CGGGGGAACT GCACCTCCAA CTTTACTCTT ACCTCCTCCG GATGCTTTTC

193_Create CACAAAACCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG
 25_Created CACAAAACCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG
 32_Created CACAAAACCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG
 15 39_Reverse CACAAAACCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG
 7_Reverse CACAAAGCCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG
 72_Created CACAAAACCT TAAGAGTTA ACTTTAGGG GAGAATTCTC TGTGGCATGG

20 193_Create AAGGATTGAG GCATTGTTGG TAAATTACCC AAACTCGAGG TCCTTATACT
 25_Created AAGGATTGAG GCATTGTTGG TAAATTACCC AAACTCGAGG TCCTTATACT
 32_Created AAGGATTGAG GCATTGTTGG TAAATTACCC AAACTCGAGG TCCTTATACT
 39_Reverse AAGGATTGAG GCATTGTTGG TAAATTACCC AAACTCGAGG TCCTTATACT
 7_Reverse AAGGATTGAG GCATTGTTGG TAAATTACCC
 25 72_Created AAGGATTGAG GCATTGTTGG TAAATTACCC AAACTCGAGG TCCTTATACT

193_Create ATCATGGAAT GCCTTCATAG GCAAGGAGTG GGAAGTAGTT GAGGAAGGGT
 25_Created ATCATGGAAT GCCTTCATAG GCAAGGAGTG GGAAGTAGTT GAGGAAGGGT
 30 32_Created ATCATGGAAT GCCTTCATAG GCAAGGAGTG GGAAGTAGTT GAGGAAGGGT
 39_Reverse ATCATGGAAT GCCTTCATAG GCAAGGAGTG GGAAGTAGTT GAGGAAGGGT
 7_Reverse
 72_Created ATCATGGAAT GCCTTCATAG GCAAGGAGTG GGAAGTAGTT GAGGAAGGGT

35 193_Create TTCCCTCACTT GAAGTTCTTG TTTCTGGATG GTGTATACAT TCGATACTGG
 25_Created TTCCCTCACTT GAAGTTCTTG TTTCTGGATG ATGTATACAT TCGATACTGG
 32_Created TTCCCTCACTT GAAGTTCTTG TTTCTGGATG ATGTATACAT TCGATACTGG
 39_Reverse TTCCCTCACTT GAAGTTCTTG TTTCTGGATG ACGTATACAT TCGATACTGG
 40 7_Reverse
 72_Created TTCCCTCACTT GAAGTTCTTG TTTCTGGATG ATGTATACAT TCGATACTGG

193_Create	AGAGCTAGTA GTGATCACTT TCCGTACCTT GAACGAGTTA TTCTTAGAGA
25_Created	AGAGCTAGTA GTGATCACTT TCCGTACCTT GAACGAGTTA TTCTTAGAGA
32_Created	AGAGCTAGTA GTGATCACTT TCCGTACCTT GAACGAGTTA TTCTTAGAGA
5 39_Reverse	AGAGCTAGTA GTGATCACTT TCCGTACCTT GAACGAGTTA TTCTTAGAGA
7_Reverse
72_Created	AGAGCTAGTA GTGATCACTT TCCGTACCTT GAACGAGTTA TTCTTAGAGA
10 193_Create	TTGCCGTAAT TTGGATTCAA TCCCTCGAGA TTTTGCAGAT ATAACCACAC
25_Created	TTGCCGTAAT TTGGATTCAA TCCCTCGAGA TTTTGCAGAT ATAACCACAC
32_Created	TTGCCGTAAT TTGGATTCAA TCCCTCGAGA TTTTGCAGAT ATAACCACAC
39_Reverse	TTGCCGTAAT TTGGATTCAA TCCCTCGAGA TTTTGCAGAT ATAACCACAC
7_Reverse
15 72_Created	TTGCCGTAAT TTGGATTCAA TCCCTCGAGA TTTTGCAGAT ATAACCACAC
193_Create	TAGCTTTAT TGATATAGAT TACTGTCAAC AATCTGTCGT GAATTCCGCC
25_Created	TAGCTTTAT TGATATAGAT TACTGTCAAC AATCAGTTGT GAATTCCGCC
20 32_Created	TAGCTTTAT TGATATAGAT TACTGTCAAC AATCTGTTGT GAATTCCGCC
39_Reverse	TAGCTTTAT TGATATAGAT TACTGTCAAC AATCTGTTGT GAATTCCGCC
7_Reverse
72_Created	TAGCTTTAT TGATATAGAT TACTGTCAAC AATCTGTTGT GAATTCCGCC
25	
193_Create	AAGCAAATTC AACAGGACAT TCAAGACAAC TATGGAAGCT CTATCGAGGT
25_Created	AAGCAAATTC AACAGGACAT TCAAGACAAC TATGGAAGCT CTATCGAGGT
32_Created	AAGCAAATTC AACAGGACAT TCAAGACAAC TATGGAAGCT CTATCGAGGT
39_Reverse	AAGCAAATTC AACAGGACAT TCAAGACAAC TATGGAAGCT CTATCGAGGT
30 7_Reverse
72_Created	AAGCAAATTC AACAGGACAT TCAAGACAAC TATGGAAGCT CTATCGAGGT
193_Create	CCATACTCGT CATTTTCA TTCCCAAGAG TGTGACAACA GTTGAAGATG
25_Created	CCATACTCGT CATTTTCA TTCCCAAGAG TGTGACAACA GTTGAAGATG
32_Created	CCATACTCGT CATTTTCA TTCCCAAGAG TGTGACAACA GCTGAAGATG
39_Reverse	CCATACTCGT CGTCTTTCA TTCCCAAGAG TGTGACAACA GTTGAAGATG
7_Reverse
72_Created	CCATACTCGT CATTTTCA TTCCCAAGAG TGTGACAACA GTTGAAGATG

193_Create ATGATGATAG TGTGACAACA GATGAAGATG ATGATGATGA TGACTCTGAG
 25_Created ATGATGATAG TGTGACGACA GATGAAGATG ATGATGATGA TGACTCTGAG
 32_Created ATGATGATAG TGTGACAACA GATGAAGATG ATGATGATGA TGACTCTGAG
 39_Reverse ATGATGATAG TGTGACAACA GATGAAGATG ATGATGATGA TGACTCTGAG
 5 7_Reverse
 72_Created ATGATGATAG TGTGACAACA GATGAAGATG ATGATGATGA TGACTCTGAG

193_Create AAAGAAGTTG CTTCTTGCCG CAATAATGTC GAGTAGTTAA GGTGTTCTGA
 10 25_Created AAAGAAGTTG CTTCTTGCCG CAATAATGTC GAGTAGTTAA GGTGTTCTGA
 32_Created AAAGAAGTTG CTTCTTGCCG CAATAATGTC GAGTAGTTAA GGTGTTCTGA
 39_Reverse AAAGAAGTTG CTTCTTGCCG CAATAATGTC GAGTAGTTAA GGTGTTCTGA
 7_Reverse
 72_Created AAAGAAGATG CTTCTTGCCG CAATAATGTC GAGTAGTTAA GGTGTTTTGA

15
 193_Create GGACTAGCCA GAGCTC.....
 25_Created GGACTAGCCA GAGCTCATGG TTTCCCGACT GGAAAGCGGG CAGTGAGCGC
 32_Created GGACTAGCCA GAGCTCG.AA TT.....
 20 39_Reverse GGACTAGCCA GAGCTCG.AA TTCACTGGCC GTCGTTTAC AACGTCGTGA
 7_Reverse
 72_Created GGACTAGCCA GAGCTCG.AA TTCACTGGCC GTCGTTTAC AACGTCGTGA

25 193_Create
 25_Created AACGCAATTA ATGTGAGTTA GCTCACTCAT TAGGCACCCC AGGCTTTACA
 32_Created
 39_Reverse CTGGGAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC
 7_Reverse
 30 72_Created CTGGGAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA GCACATCCCC

193_Create
 25_Created CTTTATGCTT CCGGCTCGTA TGTTGTGTGG AATTGTGAGC GGATAACAAT
 35 32_Created
 39_Reverse CTTTCGCCAG CTGGCGTA.. ATAGCGAAG AGGCCCGCAC CGATGCCCT
 7_Reverse
 72_Created CTTTCGCCAG CTGGCGTA.. ATAGCGAAG AGGCCCGCAC CGATGCCCT.

40
 193_Create

25_Created TTCACACAGG .AAACAGCTA TGACCATGAT TACGCCAAGC TTGCATGCCT
32_Created
39_Reverse TCCCAACAGT TGCGCAGCCT GAATGGCGAA TGGCGCCTGA TGCGGTATTT
7_Reverse
5 72_Created

193_Create
25_Created GCAGGTCGAC TCTAGCTAGA GGATCCCCGG GTACCTCTAT TGAAGAATTA
10 32_Created
39_Reverse TCTCCTTACG CATCTGTGCG GTATTCACA CCGCATATGG TGCACCTCTCA
7_Reverse
72_Created

15
193_Create
25_Created AGATCCAAGA AAAAAATGAC CCA.....AT TGCACTTCCA GAAGTCATCA
32_Created
39_Reverse GTACAATCTG CTCTGATGCC GCATAGTTAA GCCAGCCCCG ACACCCGCCA
20 7_Reverse
72_Created

193_Create
25_Created ACAATGGCAA GGCAAGCGAC ATTAAAATTG TTGAAGGAGA AAGTAAGAGG
32_Created
39_Reverse ACACCCGCT. GACGCGCCCT GACGGGCTTG TCTGCTCCCG GCATCCGCTT
7_Reverse
72_Created
30
193_Create
25_Created AAAGCCAAAG ATAGTGATTC TGAGGAGGTT GTGTCTCCTT CATTAGATCA
32_Created
35 39_Reverse ACAGACAAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA GAGGTTTCA
7_Reverse
72_Created

40 193_Create
25_Created AGACAAATAAC GAAGAACATC AGGTATTTGC TTAAAACACA ATTGTATGTA

32_Created
39_Reverse CCGTCATCAC CCGAAACGCG CGAG.....
7_Reverse
72_Created
5

193_Create
25_Created GATATTGTA TATTTGTTA GTGATATACA AAATTGTATG TAGGATATGT
32_Created
10 39_Reverse
7_Reverse
72_Created
15 193_Create
25_Created ATATTTCTG CTTACATCAC AATTGTATAT AGATATTGT ATATTTGTT
32_Created
39_Reverse
7_Reverse
20 72_Created
193_Create
25_Created AGTTATATAC AAAATTGCTT GAAAGTATATG TATATTTTT GCTTAAATCA
32_Created
39_Reverse
7_Reverse
72_Created
30 193_Create
25_Created TAATTGGATA TATATATTTG ATATCTTGGA AGTTATATAC AATAGTATGA
32_Created
39_Reverse
35 7_Reverse
72_Created
193_Create
40 25_Created ATTAAACAAT ATACAAACCT TACATTATTA TATATACAGT TAGGTACACC
32_Created

39_Reverse
7_Reverse
72_Created

5
193_Create
25_Created AAAAATTATC AAATTAAGC ACAACTTTT TATCGAATCA TATACAATT
32_Created
39_Reverse
10 7_Reverse
72_Created

193_Create
15 25_Created ATATATATAA TTGACTTAAG TAATTTATA CAACTACTTA CACTTATACA
32_Created
39_Reverse
7_Reverse
72_Created

20
193_Create
25_Created TGGGATAAGA ATTTTGCACA ATTAC
32_Created
25 39_Reverse
7_Reverse
72_Created

Table III - the protein sequences correspond to the cDNA of the auto-activators 193, 25 32 39, 7 and 72 as indicated.

	1	50
5	25 MAYAAAVTSLK RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
	28 MAYAAAVTSLM RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
	72 MAYAAAVTSLM RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
	39 MAYAAAVTSLM RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
	193 MAYAAAVTSLM RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
10	7 MAYAAAVTSLM RTIHQSMELT GCDLQPFYEK LKSLRAILEK SCNIMGDHEG	
	32 MAYAAAVTSLM RTIHQSMELT GCDLQPIYEK LRSLRAILEK SCNMMDHEG	
	51	100
	25 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
15	28 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
	72 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
	39 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
	193 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
	7 LTILEVEIVE VAYTTEDMVD SESRNVFLAQ NLEERSRAMW EIFFVLEQAL	
20	32 LTILEVEIVE VAYTAEDMVD SESRNVFLAQ NLEVRSRAMW EICFVLEQAL	
	101	150
	25 ECIDSTVKQW MATSDSMKDL KPQTSSLVSL PEHDVEQOPEN IMVGRENEFE	
	28 ECIDSTVKQW MATSDSMKDL KPQTSSLVSL PEHDVEQOPEN IMVGRENEFE	
25	72 ECIDSTVKQW MATSDSMKDL KPQTSSLVSL PEHDVEQOPEN IMVGRENEFE	
	39 ESIDSTVKQW MATSDSMKDL KPQTSSLVRL PEHDVEQOPEN IMVGRENEFE	
	193 ECIDSTVKQW MATSDSMKDL KPQTSSLVSL PEHDVEQOPEN IMVGRENEIE	
	7 ECIDSTVKQW MATSDSMKDL NPQTSSLVSL PEHDVEQOPEN IMVGRENEFE	
	32 ECIDSTVKQW MATSDSMKDL KPRTSSLVGL PEHDVEQPGN IMVGRENEFE	
30		
	151	200
	25 MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
	28 MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
	72 MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
35	39 MMLDQLARGG RELEVVASIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
	193 MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
	7 MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	

32	MMLDQLARGG RELEVVSIVG MGGIGKTTLA TKLYSDPCIM SRFDIRAKAT	
	201	250
25	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
5 28	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
72	VSQEYCVRNV LLGLLSLTSD GPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
39	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
193	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
7	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
10 32	VSQEYCVRNV LLGLLSLTSD EPDDQLADRL QKHLKGRRYL VVIDDIWTTE	
	251	300
25	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY AGSGKPPHHM RLMNFDESWN	
28	AWDDIKLCFP DCYNGSRIVL TTRNVEAAEY ASSGKPPHHM RLMNFDESWN	
15 72	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY ASSGKPPHHM RLMNFDESWN	
39	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY ASSGKPPHHM RLMNFDESWN	
193	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY ASSGKPPHHM RLMYFDESWN	
7	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY ASSGKPPHHM RLMNFDESWN	
32	AWDDIKLCFP DCYNGSRILL TTRNVEVAEY ASSGKPPHHM RLMNFDESWN	
20		
	301	350
25	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
28	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
72	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
25 39	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
193	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
7	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG SPLAITVIAG LLSKMGQRLD	
32	LLHKKIFEKE GSYSPEFENI GKQIALKGCGG LPLAITVIAG LLSKMGQRLD	
30	351	400
25	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFAIFTEDK	
28	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFAIFTEDE	
72	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFAIFTVE	
39	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFAIFTEDE	
35 193	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFATFTEDE	
7	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYFAIFTEDE	
32	EWQRIGENVS SVVSTDPEAQ CMRVLALSYH HLPSHLKPCF LYIAIFTEDE	

401

450

25 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 28 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 72 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 5 39 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 193 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 7 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR
 32 QISVNELVEL WPVEGFLNEE EGKSIEEVAT TCINELIDRS LIFIH NFSFR

10 451

500

25 GTIESCGMHD VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 28 GTIESCGMHD VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 72 GTIESCGMHD VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 39 GTIESCGMHV VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 15 193 GTIESCGMHV VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 7 GTIESCGMHD VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS
 32 GTIESCGMHD VTRELCLREA RNMNFVN VIR GKSDQNSCAQ SMQRSFKSRS

501

550

20 25 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP
 28 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP
 72 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP
 39 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP
 193 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP
 25 7 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLELGLNTWP
 32 RIRIHKVEEL AWCRNSEAHS IIMLGGFECV TLELSFKLVR VLDLGLNTWP

551

600

25 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLTISSLCYL
 30 28 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLSISSL CYL
 72 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLSISSL CYL
 39 IFPSGVLSLI HLRYLSLRFN PCIQQYQGSK EAVPSSIIDI PLSISSL CYL
 193 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLSISSL CYL
 7 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLSISSL CYL
 35 32 IFPSGVLSLI HLRYLSLRFN PCLQQYQGSK EAVPSSIIDI PLSISSL CYL

601

650

25	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRMVLK
28	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRLVLK
72	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTGNRLVLK
39	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRLVLK
5 193	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRLVLK
7	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRLVLK
32	QTFKLNLPFP	SYYPFILPSE	ILTMPQLRTL	CMGWNYLRSH	EPTENRLVLK
651					
10 25	NLQCLNQLNP	RYCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
28	NLQCLNQLNP	RYCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
72	NLQCLNQLNP	RYCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
39	NLQCLNQLNP	RYCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
193	NLQCLNQLNP	RYCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
15 7	NLQCLNQLNP	RYCTGSFFRL	LPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
32	NLQCLNQLNP	RHCTGSFFRL	FPNLKKLQVF	GVPEDFRNSQ	DLYDFRYLYQ
701					
25	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
20 28	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
72	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
39	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
193	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
7	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILRKE	IDFGGTAPPT
25 32	LEELTFRLYY	PYAACFLKNT	APSGSTQDPL	RFQTEILHKE	IDFGGTAPPT
751					
25	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
28	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
30 72	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
39	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
193	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
7	LLLPPPPDAFP	QSLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
32	LLLPPPPDAFP	QNLKSLTFRG	EFSVAWKDLS	IVGKLPKLEV	LILSWNAFIG
35					
801					
25	KEWEVVEEGF	PHLKFLFLDD	VYIRYWRASS	DHFPLYLERVI	LRDCRNLDSI
850					

28 KEWEVVEEGF PHLKFLFLDD VYIRYWRASS DHFPYLERVI LRDCRNLDI
72 KEWEVVEEGF PHLKFLFLDD VYIRYWRASS DHFPYLERVI LRDCRNLDI
39 KEWEVVEEGF PHLKFLFLDD VYIRYWRASS DHFPYLERVI LRDCRNLDI
193 KEWEVVEEGF PHLKFLFLDG VYIRYWRASS DHFPYLERVI LRDCRNLDI
5 7 KEWEVVEEGF PHLKFLFLDD VYIRYWRASS DHFPYLERVI LRDCRNLDI
32 KEWEVVEEGF PHLKFLFLDD VYIRYWRASS DHFPYLERVI LRDCRNLDI

851

900

25 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRHLFI
10 28 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRHLFI
72 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRHLFI
39 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRRLFI
193 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRHLFI
7 PRDFADITTL ALIDIDYCQQ SGVNSAKQIQ QDIQDNYGSS IEVHTRHLFI
15 32 PRDFADITTL ALIDIDYCQQ SVVNSAKQIQ QDIQDNYGSS IEVHTRHLFI

901

938

25 PKSVTTVEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~
28 PKSVTTVEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~
20 72 PKSVTTVEDD DDSVTTDEDD DDDDSEKEDA SCRNNVE~
39 PKSVTTVEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~
193 PKSVTTVEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~
7 PKSVTTVEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~
32 PKSVTTAEDD DDSVTTDEDD DDDDSEKEVA SCRNNVE~

Claims

1 A process for modifying the activation characteristics of a first polypeptide having an amino acid sequence which includes a 5 nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain

which first polypeptide mediates a cellular response leading to pathogen resistance and\or cell death or dysfunction in response to an elicitor,

10 the process comprising the step of introducing a modification to the amino acid sequence of the first polypeptide such as to produce an auto-activator polypeptide which is capable of activation in the absence of the elicitor.

15 2 A process for producing an auto-activator polypeptide which is capable of autonomous activation of a cellular response leading to pathogen resistance and\or cell death or dysfunction, the process comprising the steps of:

(i) selecting a first polypeptide having an amino acid sequence 20 which includes an NBS and an LRR domain, and which mediates the cellular response in response to an elicitor,
(ii) modifying the activation characteristics of the first polypeptide using a process as claimed in claim 1.

25 3 A process as claimed in claim 1 or claim 2 wherein the first polypeptide is an apoptosis regulator which is capable of activating an apoptosis response in a mammalian cell.

4 A process as claimed in claim 1 or claim 2 wherein the first 30 polypeptide is a resistance polypeptide which confers elicitor-dependent activation of resistance response against a pathogen.

5 A process as claimed in claim 4 wherein the resistance polypeptide is Rx or a homologue thereof.

35

6 A process as claimed any one of the preceding claims in claim wherein the modification is in an NB-ARC region of the first

polypeptide.

7 A process as claimed in claim 6 wherein the modification decreases the net negative charge of the NB-ARC region.

5

8 A process as claimed in any one of the preceding claims wherein the NBS of the first polypeptide is followed by a domain that includes any one or more of the following amino acid sequence motifs: GPLP ; CFLY ; MHD.

10

9 A process as claimed in claim 8 wherein the modification is made within an MHD and\or CFLY motif, or within less than 20, 15, 10, 9, 8, 7, more preferably 6, 5, 4, 3, 2 or 1 residue(s) of the motif.

15

10 A process as claimed in claim 9 wherein the modification is an MHD to MHV mutation.

11 a process as claimed in any one of claims 1 to 5 wherein the 20 modification is in an LRR region.

12 A process as claimed in any one of the preceding claims wherein 1,2,3,4,5, 10, 11 or more, most preferably between 3 and 11 amino acid are modified by way of addition, insertion, deletion 25 or substitution.

13 A process as claimed in any one of the preceding claims wherein the modification introduces mutations which correspond to, or are identical with, any one or more of those shown in Figure 30 3B.

14 A process as claimed in any one of the preceding claims wherein activation of the first polypeptide is mediated by elicitor-dependent dimerization,

35

15 A process as claimed in claim 14 wherein the auto-activator polypeptide comprises a dimer of the first polypeptide.

16 A process as claimed in claim 14 wherein the modification is such that the auto-activator polypeptide may be artificially dimerized under predefined conditions in response to a dimerizing effector agent which is not the elicitor.

5

17 A process as claimed in claim 16 wherein the modification comprises the incorporation of a heterologous dimerization-enabling sequence into the first polypeptide, optionally at the C-terminus and/or N-terminus, such as to permit dimerization of the 10 polypeptide in the presence of a dimerization effector agent.

18 A process as claimed in claim 17 wherein the heterologous dimerization-enabling sequence is derived from the FKBPI2 protein and the dimerization effector agent is AP20187.

15

19 A process as claimed in any one of the preceding claims, further comprising the step of screening the modified first polypeptide for its auto-activation properties.

20 20 An auto-activator polypeptide obtainable by a process as claimed in any one of claims 1 to 19.

21 An auto-activator polypeptide as claimed in claim 20 comprising any one of the sequences labelled 193, 25, 32, 39, 7, 25 72 in Table III.

22 A nucleic acid comprising a nucleotide sequence encoding the auto-activator polypeptide of claim 20 or claim 21.

30 23 A nucleic acid as claimed in claim 22 comprising a nucleotide sequence identical to any one of the sequences labelled 193, 25, 32, 39, 7, 72 in Table II.

35 24 A nucleic acid as claimed in claim 22 comprising a nucleotide sequence which is a variant of any one or more of the sequences labelled 193, 25, 32, 39, 7, 72 in Table II and shares at least 70% sequence identity therewith.

25 A nucleic acid which is the complement of the nucleic acid of any one of claims 22 to 24.

26 A process for producing a nucleic acid of any one of claims 5 22 to 24, which process comprises generating the nucleic acid via one or more PCR mutagenesis steps from a nucleic acid encoding the first polypeptide.

27. A recombinant vector comprising a nucleic acid of any one of 10 claims 22 to 24.

28 A vector as claimed in claim 27 wherein the nucleotide sequence encoding the auto-activator is operably linked to an inducible promoter.

15 29 A vector as claimed in claim 28 wherein the inducible promoter is one which is activated by a pathogen which does not provide the elicitor of the first polypeptide.

20 30 A vector as claimed in any one of claims 27 to 29 which is a plant vector.

31 A host cell comprising or transformed with a vector as claimed in any one of claims 27 to 30.

25 32 A host cell as claimed in claim 31 which is a plant cell.

33 A method for producing a transgenic plant, comprising the steps of:

30 (i) introducing a vector as claimed in any one of claims 27 to 30 into a plant cell,
(ii) causing or allowing recombination between the vector and the plant cell genome to introduce the nucleic acid into the genome,
(iii) regenerating a plant from the transformed cell.

35 34 A plant obtainable by the method of claim 33, which plant comprises the plant cell of claim 32.

35 A plant which is the selfed or hybrid progeny or other descendant of a plant of claim 34, or any part or propagule of these, which in each case includes the plant cell of claim 32.

5 36 A method of producing an auto-activator polypeptide comprising the step of causing or allowing the expression from a nucleic acid of any one of claims 22 to 24 in a suitable host cell.

10 37 A method for influencing or affecting a cellular response in a plant, which response leads to pathogen resistance and\or cell death or dysfunction in response to an elicitor, the method comprising use of any one or more of the following: the nucleic acid of any one of claims 22 to 24; the polypeptide of claim 20 or 15 claim 21.

38 A method as claimed in claim 37 comprising the step of causing or allowing expression of a nucleic acid according to any one of claims 22 to 24 within a cell of the plant.

20 39 A method as claimed in claim 37 or claim 38 for increasing the pathogen resistance of a plant, optionally by activating the resistance by contacting the plant with an appropriate inducer, which inducer is not the elicitor of the first polypeptide.

25 40 A method as claimed in claim 38 or claim 39, which method comprises any one or more ways the following:
(i) causing or allowing constitutive expression of the auto-activator polypeptide in the plant such as to activate a primary 30 resistance response which is HR independent;
(ii) causing or allowing expression of the auto-activator polypeptide in the plant under the control of an inducible promoter;
(iii) causing or allowing expression of the auto-activator 35 polypeptide in the plant from a nucleic acid construct, the accumulation of which is inhibited by the cellular response mediated by the auto-activator polypeptide;

(iv) causing or allowing constitutive expression of the auto-activator polypeptide in the plant, which auto-activator polypeptide may be artificially dimerized under predefined conditions in response to a dimerizing agent which is not the 5 elicitor, and causing or allowing expression of the dimerizing agent under the control of an inducible promoter;

41 A method as claimed in claim 38 or claim 39, comprising the steps of generating within a cell of the plant a nucleic acid 10 according to any one of claims 22 to 24 by causing or allowing the removal of a transposon sequence from within said nucleic acid.

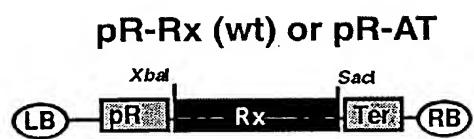


Figure 1A

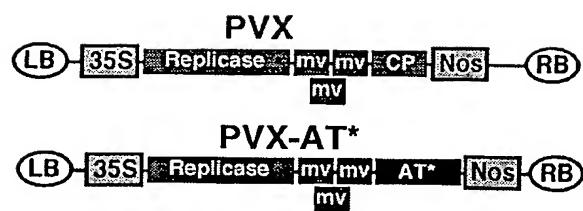


Figure 1B

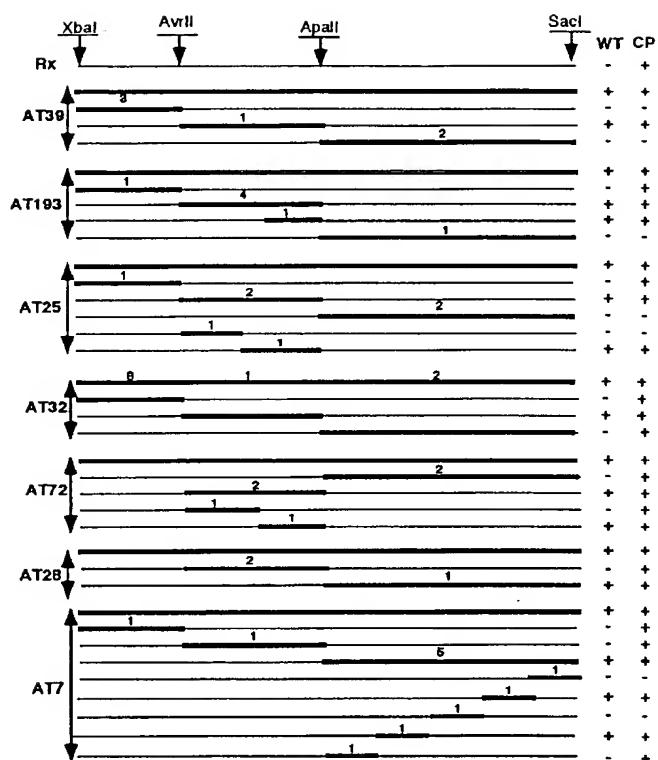


Figure 2

ARegion 1

mayaavtslmrtihqsmeltgcdlqpfyeklkslrailekscnimg
 dhegltileveivevayttedmvdsesrnvflaqnleersramwei
 ffvleqalecidstvkqwmatssdsmkdlkpqtsslvsłpehdveqp
 enimvgrenefemmlldqlarggrel

Regions 2 and 3

eVVSIVGMGGIGKTTLAtklysdpCIMSrfdirakatvsgeycvnrn
 vllgl1s1tsdepddqladrlqkh1kgRRYLVV1DD1Wtteamddi
 klcfdpcyngsrlILLTTRNveavaeyassgkpphmrlmnfdeswnl
 lhkkifekegsyspefenigkqialkcgGLPLAITVIAGLLskmgq
 rldewqrigenvvsvvstdpeaqcmrvlalsyhhlpshlkpcFLYF
 AIFTEdeqisvnelvelwpvegflneegksieevattcinelidr
 slifihnfssfrtiescgMHDVTRELCLearn

Region 4

mnfvnvirg ksdqnscaqs mqrsfksrsr
 ir ihkveelawcrnsea~~h~~s
 iimlggfecvtl
 elsfk1vrv1~~g~~lgn tw pifpsg
 vlslihlrylsrfnpclqqyqgskeavpssiidipls
 isslcylqtfklnl pfpssyypfilpse
 iltmpqlrt1cmgwny lrsheptenrlv
 lkn1qclnq1npryctgsf
 frlfpn1kk1qvgvpedfrnsqldy
 frylyqleeltfrlyypyacflkntapsgstqdplrf
 qtei1~~h~~ keidfggtapptllppp
 dafpqnlksltfrgefsavwdls
 vgk1pklevlilswnafigkewevv
 eegfphlkflf1dd vyirywras
 sdhfpylervilrdcrnld siprd
 fadittlalididyc

Region 5

qqsvvnsakqiqqdiqdnygssiev

Region 6
htrhlfipkRegion 7

Svtveddddsvttdeddddddfekevascrnnve

B

y ha	► yia	AT32
ede	► eve	AT72
deq	► dkq	AT25
h dv	► hvv	AT39 and AT193
ahs	► ars	AT7
l dl	► lel	AT7
l hk	► lrk	AT28

Figure 3

pR	LZ/NB-APC	LRR	ter	pR-Rx	-
35S	LZ/NB-APC	LRR	35 T	35S-Rx	+
pR	LZ/NB-APC	ter		pR-NBS	-
35S	LZ/NB-APC	35 T		35S-NBS	+
35S		LRR	35 T	35S-LRR	-

Figure 4

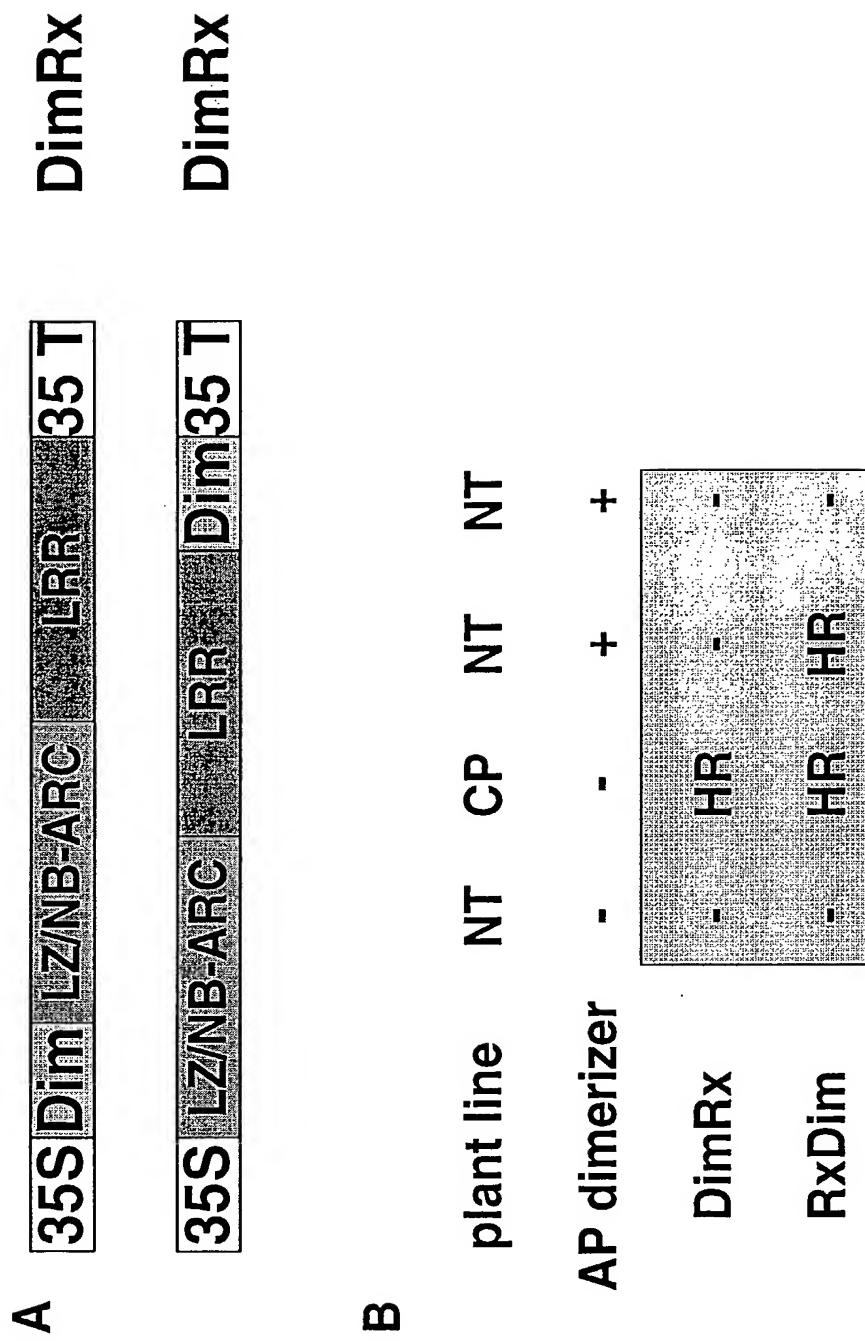


Figure 5